

Tension and relaxation in space-station science

Barring accidents, construction of the space station seems inevitable. Obstacles confront those hoping to achieve high-quality research there. There is a continuing need for close monitoring of priorities.

Do people have a future in space? This publication has already stated its view that, when it comes to science carried out on planetary surfaces, scientists are best placed in laboratories remote from the action, back on Earth (*Nature* 388, 211; 1997). There is little sign that the public is much fascinated by astronauts' activities until things start to go wrong. Although there is a role for astronauts in carrying out science on the space station, the significance of that science has been much debated (see Briefing, pages 732–737). Perhaps the only significant reason to worry about human physiology, bone decay and plant growth in zero-gravity is the certainty that sooner or later, space tourism will be upon us.

The space station's rationale and design have both changed more times than its engineers care to remember. The \$8 billion outpost that President Ronald Reagan proposed in 1984 was to have been part space factory, part laboratory, part astronomical and Earth observatory, and part transportation depot for inbound and outbound spaceships. But when the price increased by an order of magnitude and the number of missions dropped, political support eroded and the station passed a 1993 congressional vote by only one vote. It survived by being recast as a US–Russian alliance, which has presented its own difficulties.

Risks

The risks inherent in the space station are huge for the National Aeronautics and Space Administration (NASA). Troubled by burgeoning costs that have led to the scaling down of initial plans, the agency still finds itself committed to a daunting technological challenge: 45 launches over the next five years just to assemble the structure, with every reason to expect at least major unforeseen technical problems. Costs can be expected to climb, increasing the station's political vulnerability as Congress and other countries question the wisdom of continuing. And the history of technical megaprojects shows that politicians can be fickle (or, from their view, fiscally responsible) in pulling the plug abruptly, with scant concern that in doing so, huge investments (or, from their view, risky historical spending) will be wasted.

If the space station fails, it could have a chilling effect on large-scale international collaboration — certainly in the space arena, and perhaps elsewhere in science. The idea of sending more astronauts into space could be shelved, because without a station the space shuttle would have no real purpose. And a Mars adventure might be judged unfeasible if, after nearly 15 years of effort, the world's industrialized countries fail to put even a modest shelter for seven people into low Earth orbit.

These are risks that NASA's Administrator, Daniel Goldin, fully understands. For now, however, the space station has strong appeal both at the technological and the more visceral 'vision' level. The idea that Senator John Glenn should go back into space has proved to be attractive to US citizens, however sceptical scientists may be about the claim that it will help us to understand ageing. But such a stunt also reflects a questionable aspect of Goldin's approach: his readiness to cite science as a prime public justification for his activities when, in short, it

isn't. In truth, science is a figleaf for the space station's expenditure. Few scientific organizations support the station's science programme, and many have specifically opposed it — even though money saved by the station's cancellation would probably not be bestowed upon science.

Harm

There is potential harm to be done by using questionable science to sell a project that otherwise might not pass muster. It confuses a public already struggling to distinguish between scientific consensus and junk claims. It makes it more difficult for space-based experimenters — some of whom are doing interesting, if highly esoteric, basic research — to be taken seriously by their colleagues. And it risks increasing disenchantment with science if it fails.

Admittedly, once the costs of supporting and transporting experiments into orbit are factored out, the money spent on microgravity research itself is small. And whatever one thinks of the search for anti-matter, the study of dendritic structures in crystals, or the accuracy of atomic clocks (all planned experiments), the figleaf is important enough for their future to be assured in principle. But, in practice, scientists have good cause to worry.

There are already signs that space station science will not get the support researchers need to fulfil their ambitions. Some science is withering through the sheer delay in the space station schedule. Some is threatened by shortcomings in the design of the space station facilities. Another problem is that European classic: finding the money in domestic science budgets to be able to benefit scientifically from the large financial investment in facilities. Finally, quality time from the astronauts may be in short supply.

NASA — and, to a lesser extent, its partners — needs to assure scientists that this time, their basic needs will be met. On the space shuttle, scientists were promised that onboard facilities such as computers and telecommunications would be upgraded once the vehicle proved itself. But the money for such improvements never materialized, and solutions to financial or logistical problems often came at the expense of science. This tension between space-based researchers and their landlords will bear close watching.

So much for researchers involved in the space station. But what happens to others when, as they surely will, space station costs continue to soar? In the past, space science budgets have been raided. Astronomers and already beleaguered Earth-observers have good cause to anticipate further encroachments, even though the scrutiny of Congress provides some level of protection against such collateral damage.

But, barring a technological or economic catastrophe, the political and institutional momentum makes the space station unstoppable, while the US science budget is set to increase anyway. The community has placed its negative scientific judgements of the station on the record; they will have no need to feel embarrassed if the pickings are slim, and can join in the celebrations if they aren't. Whatever our feelings about the initial decision, the best the rest of us can do is relax and enjoy the spectacle. □



Industrial research booms in US, despite job cuts at large labs

[PHILADELPHIA] Despite the demise of the corporate research laboratory, industrial science in the United States is enjoying a resurgence, say research managers. It is being backed by Wall Street investors prepared to support long-term research that is on a par with the best work being done at universities and in rival companies.

Thousands of jobs have been lost at the central research laboratories of America's largest corporations over the past few years. But total research activity in US industry has been growing relatively strongly since 1994. The real growth rate of 3 per cent per year in basic and applied research is slightly less than the 4.5 per cent growth seen in total research and development.

Speakers told a symposium on fundamental research and innovation in industry at last week's annual meeting of the American Association for the Advancement of Science that the new research activity is more widely dispersed, both between corporations and within them.

"A lot of research is not being done in big laboratories, but in small clumps of business start-ups," says Rita McGrath of the business school at New York's Columbia University. "A lot of money is flowing into start-ups."

The central research laboratories of many famous US corporations have been closed in recent years, or more tightly aligned to product development. But some observers believe that the overall level of innovation is higher as a result. The central laboratories, they say, tried too hard to replicate the environment of a university — in order to recruit scientists — and failed to address the needs of the corporations paying for them.

William Brinkman, vice-president for physical sciences at Lucent Technologies, says that his was the organization "most frequently accused" of pulling back from basic research. "We have changed and I don't regret it at all," he says.

According to Brinkman, Bell Laboratories — which Lucent has operated since it split from AT&T — has returned to the emphasis on applied research which was the hallmark of its heyday. "Bell Labs in the 1950s was a very applied place," he says. By the 1970s it was far less so, "and by the 1980s the Japanese were eating our lunch".

Brinkman says that today's research operation is "very similar" to that in the 1940s and 1950s. He says the laboratories are still doing good science and that their "interactions with the business side are as good as they've been in my 30 years there".

But not everyone is happy with the pat-



Brinkman: 'no regrets' at Lucent.

tern Brinkman extols. Joseph Miller, vice-president for research at DuPont, says: "I have a great concern that many of us are walking away from innovative technology and deciding to focus on very short-term applied technology, with short-term pay-offs".

Miller is unhappy that 70 per cent of basic research in the United States is now concentrated in the universities: he believes that technology often stimulates science, and that industry must drive this itself. "There are limitations to academic research as an alternative to industrial research."

Miller says government statistics indicate that research spending by US industry "has levelled off". But Chuck Larson, director of the Industrial Research Institute (IRI), a Washington-based organization supported by large corporations, says Miller's argument is based on outdated official statistics from the National Science Foundation (NSF).

"Often you hear that industry is farming out its innovation — but there has been a dramatic change in the data since the last NSF numbers were compiled," Larson says. IRI collects information more quickly — and reports that basic and applied research in industry has been increasing by 3 per cent a year in real terms since 1993. "I talk to a lot of people, and [large corporations] are doing as much fundamental research as ever."

Different sectors have, of course, taken different approaches. In the oil industry, where prices have fallen to pre-1974 levels of around \$16 a barrel, the retrenchment in corporate research may have changed atti-

tudes for good. Ed Witterholt, innovation hunter for BP, the largest oil producer in the United States, says: "If the oil price went back to \$35 a barrel and someone said 'let's start doing big research programmes like we did before', we wouldn't do it, because we've changed our religion."

These days, BP depends on suppliers such as Schlumberger for technological innovation. In its own business of oil exploration and production, it collaborates with once-feared rivals Chevron, Mobil and Texaco.

As Witterholt points out, 300 such collaborative arrangements have been registered with the Justice Department under a provision that exempts "pre-competitive research" from the strict anti-trust laws that normally stop US corporations from working too closely with their competitors.

Business schools and consultants, meanwhile, are telling research managers to frame their operations in terms that investors can understand. Herbert Eleuterio, a former US research manager now at the National University of Singapore, where he advises the government on innovation, says: "You need people with backbone to stand up and defend high-risk, high-reward projects." Philip Brodsky, vice-president for corporate research at Monsanto, says: "You have to have a powerful vision that you can convey to a stock analyst."

Neither of them accepts that short-term investors are selling industrial research short. "The health of the global industrial enterprise is robust," says Eleuterio. He adds that industry's main concern should be optimizing its links with government-funded research. As for its own efforts, he says: "I'm very optimistic; I think the private sector is going to do the right thing." **Colin Macilwain**

Germany approves DNA tests for visas

[MUNICH] Germany has agreed to accept DNA tests as proof of a blood relationship in visa applications. According to government officials, the move is intended to help Kurds from Turkey and Iraq who wish to join relatives granted political asylum in Germany but are often unable to produce documents acceptable to the authorities.

The idea was put to the German foreign ministry by the country's embassy in Ankara, where increasing numbers of Kurds have been applying for visas in recent months. The embassy began accepting DNA tests as proof of relationship last December. Since then, about 25 visa applicants have

taken up the option. Applicants can choose whether to present documents or the results of a DNA test.

The test uses saliva samples from the visa applicant and his or her relative in Germany, and is carried out privately at the Institute for Forensic Medicine in Münster. Applicants must pay the DM600 (US\$332) cost.

Data from the test remain confidential between the institute and the applicant. "We can't find any ethical objection to this procedure," says Dirk Lanzerat, a biomedical ethicist from the Institute for Ethics in Science in Bonn. **Burkhardt Roeper**



Going up: the Gemini facility at Cerro Pachon, Chile, pictured last month.

Seventh heaven for Australia's optical astronomers

[CANBERRA] The Gemini optical telescope project has broadened its international membership by including Australia as the seventh partner, alongside the United States, the United Kingdom, Canada, Chile, Argentina and Brazil.

The announcement, due to be made yesterday (18 February) by the education minister, David Kemp, ends several years of frustration for Australian astronomers who missed an earlier opportunity to join Gemini and were also denied the chance to join the Very Large Telescope project in Chile.

Australia will contribute A\$13.5 million (US\$9.2 million) over five years towards Gemini's capital costs, plus A\$667,000 a year in running costs (rising to A\$1.1 million in 2001 and beyond). This is one of the largest allocations of public money Australia has made to a basic research facility. Although the money is not 'new', having been designated as a priority in the Australian Research Council (ARC)'s budget, it shows a fresh willingness by the government to support a flagship project with popular appeal.

Gemini operates a pair of telescopes in Chile and Hawaii. Matt Mountain, director of Gemini within the US National Science Foundation, says the extra funds will allow "enhanced scientific productivity". This will offset the small decrease in the original partners' observing time needed to accommodate Australia's share. Forthcoming technical enhancements will include infrared sensors to see guide-stars in the brighter hours of dawn, allowing extended observing time.

Australia was invited in 1994 to join the Very Large Telescope project of the European Southern Observatory in Chile, but in 1996 the then science minister, Peter McGauran, finally refused to finance Australia's entry (see *Nature* 381, 100). The UK Particle Physics and Astronomy Research Council later backed an ARC bid to persuade the Gemini partners to expand their membership. Securing funding remained problematic but now seems to have been achieved.

Peter Pockley

Moscow's 'missing fossils' come under new scrutiny

[MUNICH] A criminal investigation may soon begin into the alleged theft of fossils said to be worth a million US dollars from the Palaeontological Institute in Moscow, part of the Russian Academy of Sciences.

Yuri Rybakov, an independent member of the Russian parliament, has said that he is convinced by evidence from some of the institute's researchers that its management had been involved in illegally approving the export and sale of items from its fossil collection, one of the most important in the world.

Since 1992, 27 labyrinthodont skulls, including three holotypes (reference specimens), five dinosaur skulls, six mammoth tusks and a collection of ammonites have gone missing. Some have subsequently been identified in collections in the West. The full list of missing fossils may be even longer than this, say the institute's scientists.

The scientists have until now been afraid to speak out for fear of losing their jobs. But the directors of the institute have started reducing staffing levels in anticipation of the academy's general restructuring programme. And some of those who have lost their jobs have decided to make the thefts public.

Last autumn the director of the institute, Alexei Rozanov, announced a plan to cut half of the scientific staff positions. Although he backed down after legal action by the unions, Rozanov announced shortly before Christmas that ten per cent of the posts were to go, based on a recommendation expected from the academy. The brunt of the cuts has fallen on researchers rather than administrators, including some of those most concerned about the missing fossils.

In January, seven senior scientists from the institute sent an open letter to the All-Russian Palaeontological Society, outlining their fears for the collection, and pointing out that the authorities had "kept silent about" the missing fossils, rather than investigating them properly. The letter also questioned the decision to sever the institute's connections with the international working group that had been set up two years ago to help locate and return stolen fossils (see *Nature* 384, 499; 1996), and brought into question the reasoning behind the recent sackings.

It also refers to the secrecy surrounding Nauka (Science), a company recently created within the academy of sciences. The Palaeontological Institute signed an agreement with Nauka in December, giving the company responsibility for its exhibitions of fossils and other commercial activities. Scientists fear 'covert' privatization of the fossil collection, and suspect that parts of it might be sold off.

Vladimir Strakov, director of the Institute

of Earth Physics in Moscow and a member of the Guild of Directors of the Academy of Sciences, believes all these issues are linked. He is also concerned that the academy has been reluctant to address what he described as the "deep problems" of the institute.

Results of a limited inquiry into the missing fossils set up by the academy last year were never made public. The head of the inquiry committee, Lev Andreev, director of the academy's Botanical Gardens, says that there was "no evidence of deliberate theft — aside from one skull which has now been returned — but only the absence of a properly organized inventory system". After the investigation, the academy gave the institute money to improve its security system, he said.

Strakov has recently taken up the case of the missing fossils, and says that even though he anticipates coming under pressure from the academy — and could lose his job under cover of the academy's reorganization — he will fight "to a conclusion". Strakov is particularly concerned that the substantial earnings from the institute's recent exhibitions abroad appear not to have filtered back into the institute as had been intended.

The science committee of the Duma, the Russian parliament, is considering his request for a parliamentary inquiry into the problems of the institute. Strakov says that his biggest concern is to stop the sacking of scientists. He says that the Palaeontological Institute has been acting prematurely in cutting staff, pointing out that none of the institutes in his branch of the academy — geology, geophysics and mining engineering — has yet started making people redundant.

The directors of the institute have declined to comment on the situation, pleading illness. They have also twice failed to turn up in court to defend themselves against an appeal for reinstatement and compensation by a former employee, Masha Hekker, who was sacked after being accused of "truancy from work". Hekker says she had received formal permission for a three-month leave of absence to carry out research in Brussels. Her case is being supported and financed by the International Commission on Human Rights.

Alexander Zhamoida, vice-president of the All-Russian Palaeontological Society, has replied to the scientists in an open letter. It agrees on the need "to prevent illegal actions" relating to the theft of fossils and other items of national heritage, but says that some members of an international working group for the return of the fossils "purposefully intend to discredit the Palaeontological Institute". It adds that "those who signed the letter are not acting with the noblest aims".

Alison Abbott

Clinton picks NSF chief as science adviser

[PHILADELPHIA] Neal Lane, the director of the National Science Foundation (NSF), is to succeed Jack Gibbons as science adviser to President Bill Clinton and director of the White House's Office of Science and Technology Policy (OSTP).

The announcement of the appointment, which ends 18 months of sometimes fanciful speculation about who would succeed Gibbons, was made by Clinton during his address on 13 February to the annual meeting of the American Association for the Advancement of Science (AAAS) in Philadelphia. It was the first speech made by a sitting president at the meeting since Harry Truman spoke there 50 years ago.

Clinton also said that Rita Colwell, currently director of the biotechnology institute at the University of Maryland at College Park, will succeed Lane as director of the NSF. Colwell, whose main research interest is the study of marine microbes, was nominated last month to serve as Lane's deputy, and now becomes the first life scientist — as well as the first woman — to lead the NSF.

Speaking immediately after Clinton's announcement, Lane said he was "very honoured" to succeed Gibbons. Asked why he was ready to accept a job that is widely regarded as a lot of trouble, Lane said simply: "I'm interested in the whole of the scientific enterprise, and the president asked me."

But he added that the offer of the post had not come directly from Clinton. "It was conveyed to me," he said. "I'd been talking to the vice-president, and of course today I got the opportunity to meet with the president. I'm hoping now to build on the work that Jack Gibbons has done."

The appointment has already triggered speculation about how the mild-mannered Lane will fare in the hot-house atmosphere of Clinton's White House. "It'll be interesting to see how he gets on," says one scientist who recently served under Gibbons at OSTP. "I wouldn't have thought it'd be his style, because he's basically a quiet guy."

But defenders of Lane, who worked as a physics professor and then provost of Rice University in Houston, Texas, before becoming director of the NSF in 1993, say that he has learned to operate effectively in Washington in the five years since then.

Lewis Branscomb, a former chairman of the National Science Board and now at Harvard University, said he thought Lane would do well. "If you look at how he's led NSF, he's been cautious, careful and skilful. He's made NSF's role clear to the Congress."



Science fan: President Clinton addresses the AAAS meeting, the first sitting president to do so in 50 years. In addition to announcing new appointments, he praised fusion and AIDS research.

Branscomb added that some of the earlier speculation that a "heavy hitter" such as John Deutch, the former deputy defence secretary, or Norman Augustine, the chief executive of Lockheed Martin, might take the OSTP post was "just ridiculous", as the job lacked the clout needed to attract such people.

More support for Lane came from Chuck Larson, director of the Industrial Research Institute. "It is the perfect match, under the conditions we have," he said. "We were looking for an industrial person, such as Norman Augustine, but it's tough to get someone like that. Neal has fought for NSF's budget, he's worked with the Congress, and he'll get on with the president."

Clinton's address to the AAAS was low key and repeated points made in his recent State of the Union address and in other recent speeches about science (see *Nature* 391, 521; 1998). The president, who is being

relentlessly hounded over allegations of marital infidelity, looked tired, although he appeared to be reinvigorated as he mingled after the meeting with some of the 2,500 scientists and schoolchildren who came to hear him.

One man who was pleased to shake his hand was Dale Meade of the Princeton Plasma Physics Laboratory in New Jersey, as Clinton had endorsed the fusion programme in his speech, predicting that in 50 years' time "fusion and solar power may yield abundant energy". Clinton also praised progress in AIDS research: "If this progress continues, I believe we'll have an effective vaccine within a decade," he said.

Lane needs security clearance, as well as Senate confirmation, before he can take up the position, and this could take two or three months. Gibbons will leave the post on 15 March.

Colin Macilwain

NIH peer-review revision panel is named

[WASHINGTON] The US National Institutes of Health (NIH) last week announced the 15 members of a 'blue ribbon' panel charged with exhaustively reviewing — and making recommendations for changing — the structure of its peer-review system.

The panel will spend the next year studying the organization of some 100 study sections and deciding whether they reflect current science or have become anachronistic. It is expected to advise on whether a broad reconfiguration of sections is needed, or whether regular monitoring is sufficient to keep the composition and subject areas of sections up to date.

Prominent members of the Panel on Scientific Boundaries for Review include Bruce Alberts, the president of the National Academy of Sciences, Stuart Orkin of Harvard Medical School and David Botstein of the Stanford University School of Medicine. The chair of the panel will be appointed at its first meeting, which is unlikely to take place before April.

Alberts, who has chaired a study section, says that many of the study sections represent "ancient ways" of dividing up the field, and that some form of change is needed. He favours broader study sections that look for innovation, and would also

like to see close scrutiny of ossified sections that may have become virtual entitlement programmes for their "regular clientele", while other sections in cutting-edge areas are overwhelmed with deserving applications.

Elvera Ehrenfeld, director of NIH's Center for Scientific Review, who appointed the panel as part of a broad reassessment of NIH's peer-review system, says she is open to all ideas that aim to keep peer review abreast of science. "The process needs to be driven by science, and not the other way around," she told the advisory panel to Harold Varmus, director of the NIH, in December. Meredith Wadman

NSF



Lane: seen as an effective operator.

Gene marker database stirs up debate

[WASHINGTON] The US National Cancer Institute (NCI) is launching a project to identify, catalogue and enter into the public database its own set of single-nucleotide polymorphisms (SNPs, or 'snips') — single-base variations in human DNA that are useful genetic markers.

The NCI's 'genetic annotation initiative', which comes hot on the heels of a similar project recently launched by the National Human Genome Research Institute (NHGRI), will seek out SNPs that mark 1,000 cancer-related genes, from tumour-suppressor genes to genes involved in metabolism and DNA repair.

The project, which also involves the National Center for Biotechnology Information, will receive \$1.6 million this year, and planners have already chosen 300 of the 1,000 genes to be studied.

But the sample set that the NCI is using to launch the project — DNA from 10 individuals in the collection of Paris's Centre d'Etude du Polymorphisme Humain (CEPH) — has raised eyebrows at the NHGRI, which last year launched its own massive effort to identify and make public 100,000 or more SNPs.

The genome institute's samples will come from up to 500 ethnically diverse Americans, including those of Asian, Native American, African and European descent. The CEPH collection, by contrast, is dominated by Caucasian samples from more than 60 extended families originally collected by researchers at the University of Utah for genetic mapping studies. Most polymorphisms are thought not to be specific to ethnic groups.

Richard Klausner, the NCI director, described the project at a meeting of the NHGRI's advisory council last week. But NHGRI's director, Francis Collins, told him



All sorts: critics claim the NCI's collection of SNPs will be too limited to Caucasian samples.

that he had "a little trouble with the notion that you're going to automatically overlook polymorphisms that are present in other ethnic groups than Europeans".

Collins said he feared that "going back later" to find SNPs in ethnically diverse groups will be "less attractive" to researchers than if SNPs were first identified in an ethnically mixed set of samples.

Collins also argued that, in creating a database using different samples from those being assembled by the NHGRI, the NCI would prevent a desirable situation in which "polymorphisms that are being discovered by groups all over the place will be attached to the same anonymous DNA samples".

Klausner responded that ethnically diverse groups can be examined in second- and third-generation studies once a relevant set of SNPs are identified. "I don't disagree with the importance of [studying diverse

samples]," Klausner said. "It's just a question of whether it actually does need to be there as the up-front issue."

For Klausner, the project's most urgent goal is to identify SNPs, to make them accessible to researchers quickly, and to study which technologies are best at doing this — a question that remains unanswered.

Ken Buetow, who heads the human genetics programme at Fox Chase Cancer Center in Philadelphia, has been enlisted by Klausner to oversee the initiative with Michael Dean, chief of the human genetics section at the NCI's Laboratory of Genomic Diversity.

Buetow says that planners aim rapidly to study a second set of ethnically diverse samples, including some from the NHGRI's incipient database. But he says that initially there are compelling scientific reasons for using the CEPH collection. Because the collection includes large extended families, SNPs that are discovered can be rapidly validated through tracing their inheritance, ensuring that they are not laboratory artefacts.

The fact that the CEPH collection has already been extensively genotyped will allow investigators readily to fit identified SNPs into an existing map, as well as helping them to detect errors more easily.

But that does not satisfy everyone. "We would rather do all of these kinds of studies on a common set of DNAs," says Aravinda Chakravarti, a professor of genetics at Case Western Reserve University in Cleveland who heads an NHGRI working group that is planning that institute's SNP project. He adds that it would be "much more helpful" to look at "a diversity of types from multiple ethnic groups", such as those in the NHGRI sample group.

Meredith Wadman

Quebec urged to match supply and demand for science graduates

[MONTREAL] An alliance of Quebec organizations called Science pour Tous (Science for All) is proposing that high-technology companies should set aside 1 per cent of the public funds they receive to promote public understanding and appreciation of science.

The alliance was formed last November by seven organizations, alarmed at the growing shortage of trained scientific and technological personnel. A manifesto signed by 43 prominent educators, scientists and other public figures says that science and technology are not treated with due importance by Quebec institutions. It calls on scientists to share with the public "their questions, their discoveries, their sense of wonder, and even their distress".

A meeting of l'Association de la Recherche Industriel du Quebec heard that businesses are searching desperately for graduates in information technology, biotechnology and aeronautics. The province has recently produced fewer science graduates, but employment opportunities in these areas have increased by 91 per cent during the past 10 years, compared to 14 per cent in other sectors.

Despite this, the provincial education minister has reduced the number of hours devoted to the sciences in secondary schools and the federal government has announced the abolition next year of a programme called Science Culture Canada. This programme has provided up to C\$2.5 million (US\$1.7 million) a year to non-profit

groups that stimulate interest in science and technology among young people.

"With several hundred thousand dollars invested in Quebec, this programme has aided enormously in the promotion of science among young people," says Michel Gauquelin, vice-president of Science pour Tous and director general of the popular science magazine *Quebec Science*.

The programme was a victim of 1995 federal budget cuts, and is to hold its last competition for funds in April — despite the recommendation in a 1993 study by a consulting firm that it should be continued. The initial response to Science pour Tous from the Quebec government's ministry of culture and communication has been positive, says Gauquelin.

David Spurgeon

Tensions grow over access to DNA bank

[PARIS] A debate about the commercial use of DNA banks seems about to erupt in France following controversy about a deal giving the French biotechnology company Genset access to the 'Chronos' DNA bank. The bank contains samples of DNA taken from centenarians and was built up for research into longevity at the Jean Dausset Foundation—Centre d'Etudes du Polymorphisme Humain (CEPH), a non-profit-making organization based in Paris.

The controversy has been triggered by the dismissal in November of François Schächter, a geneticist at CEPH who worked on the bank's creation in 1991 and its subsequent development. A CEPH spokesperson said there had been a loss of confidence between Schächter and the management, and alleged that Schächter had not respected the terms of confidentiality stipulated in the contract with Genset.

But Schächter, who plans to challenge his dismissal in an industrial tribunal, is expected to argue that his sacking stemmed from his opposition to the Genset deal and the way in which confidentiality clauses in the agreement hampered his collaboration with groups elsewhere, and that his eviction from CEPH denied him access to his research materials.

Under a contract agreed between CEPH and Genset in 1996, the company has a first right of refusal on results from CEPH's research into ageing, including the Chronos bank, for three years, in return for funding of CEPH and the ageing programme in particular of FF32 million (US\$5.3 million). The contract requires CEPH to make Genset an offer before ceding any DNA collections associated with the programme to a third party.

The contract also forbids the passing of any information about research into ageing to a third party — including oral presentation at conferences — without Genset's authorization. Internal CEPH rules forbid staff to divulge "any" form of information without authorization from management.

Gilles Thomas, who was appointed scientific director of CEPH in 1996, says the sacking was unavoidable, alleging that Schächter refused to cooperate with management and infringed confidentiality requirements. Thomas also denies that the management inhibited collaboration, claiming the decline in output from Schächter's laboratory was the result of poor productivity.

CEPH was previously involved in controversy about its DNA banks in 1994, when Philippe Froguel, then a CEPH researcher, successfully challenged plans by CEPH to give exclusive access to a DNA bank constructed by his group for diabetes research to Millennium, a US biotechnology company (see *Nature* 368, 175; 1994).

A panel set up by the French government



Ageing genes: until her death last year aged 122, Jeanne Calmant was the world's oldest woman.

then recommended that bodies that paid for the DNA bank should have the right to open it to a third party. But it added that the researchers who built the bank also deserved rights, and should have a period in which to exploit the bank themselves (see *Nature* 370, 4; 1994). It also said that donors must agree to any use of such DNA — in particular to commercial applications — and that international agreements were needed to ensure open access to all DNA sequence databases.

But Axel Kahn, a member of the panel, points out that its recommendations were not translated into law, as originally intended. The conditions under which Schächter was ousted from CEPH "run against" the spirit of the recommendations in that it is "obvious" that the scientist has a right to exploit the bank, says Kahn. He is also critical of what he describes as the lack of openness surrounding the negotiation of the CEPH–Genset contract. "A moral engagement was made with the people sampled, which demands that they be informed of the commercial exploitation of their genes," he says.

Michel Allard, scientific and medical director of the IPSEN Foundation, a Paris-based body that helped to organize the campaign to persuade centenarians to contribute their DNA to Chronos, says he now feels "betrayed". "We never told the centenarians that their genes might be patented," he says. "I am not against a contract with a third party, but it should have been done in an open way," says Allard, who also protests that IPSEN was not involved in CEPH's discussions with Genset over the bank.

The contract between CEPH and Genset requires both parties to respect the recommendations of the Louisot report. Thomas disputes Schächter's rights to access the bank, claiming that he was only one of several researchers involved in creating the bank, not the principal instigator. But Allard and others contest this, arguing that Schächter was the driving force behind its creation.

Thomas says that CEPH is willing to discuss giving Schächter access to the bank "if Genset wants to [discuss a collaboration]".

Schächter, who was the first author of one of the first papers showing evidence of differences between centenarians and the rest of the population in the frequency of alleles involved in some age-related diseases (see *Nature Genetics* 6, 29; 1994), has received backing from many colleagues.

In November 11 national coordinators of an EU network on "molecular gerontology" wrote to Jean Dausset, the president of CEPH, protesting that the deal with Genset had "seriously hindered" Schächter's participation in the network, and that it had "a serious negative impact on the progress of the EU Biomed-funded initiative".

They add that, while appreciating the need to protect sensitive commercial information, "in this instance, a basic principle of our scientific community, freedom of research seems to have been compromised".

"We have missed the input of Schächter," says Tom Kirkwood, director of the biological gerontology group at the University of Manchester, one of the signatories of the letter. He describes Schächter as a "very talented and imaginative young scientist" and the creation of the Chronos bank as "ground-breaking". It contains DNA samples from hundreds of centenarians, including Jeanne Calmant, who was the world's oldest woman until her death last year at the age of 122.

Similar banks have since been established worldwide, says Kirkwood, adding that "France took a lead in longevity research, but



Schächter: sacking started debate.

the country's reputation is now being damaged". But Thomas dismisses such protests, alleging they are based on Schächter's account of events, and that he solicited some directly. He also denies that the rules on confidentiality inhibited collaboration. Schächter last week declined to comment publicly on the grounds that litigation is in progress. Genset officials were unavailable for comment.

In a separate development, CEPH is embroiled in a legal dispute with Genset about what it claims is the latter's refusal to pay instalments of the FF32 million to CEPH as agreed in the contract.

Thomas declines to comment on the reasons for Genset's refusal on the grounds that legal proceedings are under way. But he denies that CEPH has reduced its own funding for research into ageing, which would have infringed the contract.

Declan Butler

India to challenge Basmati rice 'invention'

[NEW DELHI] India has been dragged into another dispute with the US Patents and Trademark Office, this time over the patenting of 'Basmati rice', the long-grain rice variety known for its distinct aroma and unique to the Indian subcontinent. It has only recently won a patents case over the plant turmeric (see *Nature* 389, 6; 1997).

The US seed company RiceTec, based in Alvin, Texas, claims to have "invented novel rice lines" whose grains have qualities of Indian Basmati and can be grown worldwide. US patent 5663484 would allow the company to market its aromatic rice as Basmati.

India says the US company is free to patent its aromatic rice under any name except Basmati, which it says is specific to the varieties grown for centuries by farmers in northern states of India and parts of Pakistan. It argues that the name Basmati cannot be used to describe another product, even if it is similar to the Indian variety. But RiceTec claims that "Basmati is a generic name for a type of rice just as durum is for a type of wheat". It has dismissed Indian claims about uniqueness, saying aromatic rice grows worldwide.

RiceTec president Robin Andrews says the US patent is for an improved version of the "long-grain American Basmati rice" his company has already been selling under the brand names 'Texmati' and 'Kasmati'. Paula Coute, a spokesperson for the US patents office, says the RiceTec strain



Rice row: Basmati name in dispute.

Call for moratorium on seedbank patents

[LONDON] The United Nations Food and Agriculture Organization (FAO) is to consider a moratorium on patent applications for original varieties of seeds held on behalf of the international community by a network of international agriculture research centres.

The seeds are held in seedbanks run by the Consultative Group on International Agriculture Research (CGIAR), a network of research institutes mainly in the developing world but operated through the World Bank in Washington DC.

The call for the moratorium was made by

CGIAR, and will be considered at a meeting in June of the FAO's commission on genetic resources. It comes in the wake of attempts by research institutes in Australia to file claims to intellectual property rights on seeds borrowed from CGIAR seedbanks in India and Syria.

The patent applications were discovered by the environmentalist group RAFI (Rural Advancement Foundation International), which alerted CGIAR and the FAO. The applications have since been withdrawn.

Pat Mooney, executive director of RAFI, welcomed

the call for a moratorium. But plant breeders are sceptical. Bernard Le Buanec, secretary general of the International Plant Breeders' Association in Switzerland, questions the need for a moratorium on the grounds that international law already forbids the patenting of original varieties of germ plasm.

Geoffrey Hawtin, director general of the International Plant Genetic Resources Institute in Rome, says Le Buanec is "technically correct" but a moratorium will prevent patents being filed on original varieties of seed in countries where this is not illegal.

Ehsan Masood

deserved to be called Basmati as it met the definition of Basmati.

But Indian officials say the patent would have a heavy impact on India's exports of Basmati, worth \$800 million a year. "What is worse is the negation of centuries of contributions by traditional farmers who have bred and cultivated Basmati," says Vandana Shiva, an expert in biodiversity with the environmentalist group Third World Network. She says real Basmati will now have to compete with "fakes" and substandard Basmati grown outside the subcontinent — a possi-

bility that must worry consumer groups.

The Indian government has promised that the patent will be challenged. A high-level inter-ministerial group, including M. S. Swaminathan, the former director-general of the International Rice Research Institute in Manila, has been set up to present the country's case to the US patent office. Several approaches are being considered.

Scientists say one approach is to establish, through DNA fingerprinting, that the source of RiceTec's variety is the germ plasm from India accessed through international gene banks. "No one can breed Basmati without using original lines from us," says Suresh Sinha, former director of the Indian Agricultural Research Institute (IARI) in New Delhi.

Another approach being considered is to see whether RiceTec's patented variety measures up to the quality standards laid down for Basmati rice in India. E. A. Siddiqi, who has worked on the genetic improvement of Basmati for more than 20 years at IARI, says Basmati rice must pass seven tests.

India is also likely to initiate a dispute at the Geneva-based World Trade Organization. Suman Sahai, a geneticist and president of Gene Campaign, a non-governmental organization, says India and Pakistan should take the case to the organization's dispute settlement board on the grounds that patenting Basmati violates the protection offered to products that are 'geographically indicated' under the TRIPS international trade and intellectual property rights agreement.

Sahai claims that Basmati rice is exclusively associated with India and Pakistan in the same way as champagne is associated with France.

K.S. Jayaraman

'Give us back our mansion,' plead scientists

[MOSCOW] A Russian court has partially complied with a complaint lodged against President Boris Yeltsin by scientists in the Theoretical Problems Department of the Russian Academy of Sciences. The scientists have been demanding the right to return to a property in Moscow from which they claim they were unlawfully removed.

In 1982 the academy built flats for 26 families formerly living in the building, and made repairs so that it could be occupied by a group of scientists headed by Professor Erast Adriankin. Yeltsin confirmed in 1991 through a decree that the mansion should belong to the scientists forever.

In January 1996, however, Yeltsin signed a new decree, according to which the building would be made available to official bodies, including representatives of the Chechen Republic. The scientists refused to leave and were removed by force. Their research programmes were disrupted.

The scientists took their complaint to an arbitration court, and in February 1997 Yeltsin's decree was cancelled. But the chairman of the court, Veniamin Yakovlev, disagreed with this decision, and the case was sent back for further investigation.

"We have been compelled to work at home without access to the documents and equipment left at our department," Adriankin said at the second court hearing. "We know that many of these have disappeared or been damaged. For two years we have not been receiving salaries, although we are working on important projects in space research and in research related to destruction of chemical weapons."

The court has now suggested that the department should get back just over a quarter of its former mansion. But the scientists remain dissatisfied. Adriankin says: "We shall continue our attempts to get back what belonged to us."

Carl Levitin

A degree of success for MRes courses

[LONDON] A controversial one-year post-graduate degree designed to train students in research skills has been praised by students, universities and research councils in the United Kingdom, defying widespread scepticism that the course would fail to take off.

The idea of a 'research master's' degree (MRes) received a lukewarm response when suggested by the former Conservative government in its white paper on science and technology policy in 1993. But with the pilot phase now into its third year, several universities have acknowledged that the courses provide a good preparation for a PhD programme, and last week the government said it will allow sponsoring research councils to continue to fund the course when the pilot phase ends this summer.

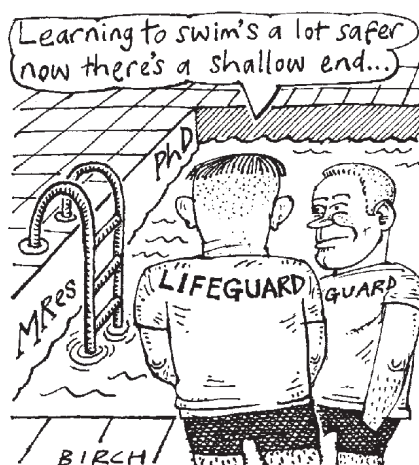
Several universities say they have been surprised by the large number of high-calibre students that many MRes courses tend to attract. This trend was confirmed in a report from the four research councils that have been supporting the degree. "We were all a bit sceptical at the start," says Bob Price, director of human and corporate resources at the Biotechnology and Biological Sciences Research Council, one of the sponsoring councils. "No one was totally enthusiastic."

Not everyone has been convinced. The Particle Physics and Astronomy Research Council and the Institute of Physics, for example, remain sceptical about the appropriateness of the MRes approach. They argue that the degree's structure — short project work in one or two subject areas combined with taught courses in research skills — will not be directly relevant to the longer-term research of a PhD.

Philip Diamond, manager of higher education and research at the Institute of Physics, says that the four-year MPhys degree courses supported by the institute are a better preparation for research in physics. About one-third of UK undergraduate physics students are studying for an MPhys degree.

But Price says that most of the universities taking part in the project have been convinced of the degree's uses as an introduction to research skills. Some are even offering MRes programmes funded through non-government sources, and many receive students from overseas.

The MRes course can be likened to a 'PhD primer'. Students spend two-thirds of their time undertaking several short, or one longer, research project. The remainder is spent studying taught courses on research skills. The short projects are in a different subject from the student's first degree. The idea is to show that research skills need to be transferable, as full-time researchers often



have to change fields or work in multidisciplinary teams.

The origins of the course stemmed from concern that some students embarking on three-year PhD programmes lacked the confidence or ability to study independently, leading them to drop out. An MRes course would allow them to assess whether or not they were suited to research.

If at the end of the course they choose not to continue with a PhD, they would have a

qualification that would also be relevant to industry. Chris Wharton, co-director of the MRes programme at the University of Birmingham, says if he had his way he "would get all PhD students to do this course".

Wharton says that one of his main worries when the course began was whether it would attract enough high-calibre applicants. This fear appears to have been unfounded. The research council report shows that last year 32 per cent of MRes applicants in the biological sciences came with first-class degrees, compared with just 11 per cent of applicants for conventional master's degrees.

The research councils have supported more than 200 students per year on 30 courses at various UK universities. Courses offered by the medical, natural environment, and biological sciences councils are oversubscribed. In contrast, the Engineering and Physical Sciences Research Council — ironically one of the most enthusiastic supporters of the MRes — is having difficulty attracting suitable students, as a relatively high proportion of those ending their course have been undecided about their future career.

Ehsan Masood

Spain lifts block on Framework funds

[MUNICH] The European Union's next five-year Framework programme of research (FP5) has cleared another hurdle in its problem-strewn path towards approval. Contrary to widespread fears, the council of research ministers reached a 'common position' on the European Commission's proposal last week, as Spain was persuaded at the last moment to lift its threatened veto.

This was an important step because of the tight timetable. Second readings in parliament and council are still required, but the programme must be adopted well before the end of the year to ensure that there is no gap in funding for researchers (see *Nature* 391, 519; 1998).

In its bid to avoid such a gap, the council has had to make a compromise that may cause problems later. Spain is refusing to approve any programme to be financed under the European Union's financial arrangements for the period beyond 2000, whose terms are being negotiated. It is using this weapon to try to force ministers to agree to maintain the union's generous subsidies to Spain after 2000. To sidestep the problem, the council's proposal approves Framework funding only for 1999; continuing funding will depend on the negotiations.

Assuming that funding is continued as planned, the budget of the council's common position is only ECU14 billion (US\$15.2

billion). This figure drew immediate angry responses from both commission and parliament which had put forward ECU16.3 billion and ECU16.7 billion respectively.

Research commissioner Edith Cresson referred to the council's meeting as "black Thursday". She said the funding proposed, which would be less in real terms than the funding for the current five-year programme, was a "negative signal" to industry. She contrasted the council's common position with recent promises of generous increases in US funding for research.

Parliament, which has co-decision rights with the council, will almost certainly reject such a low level of funding, and a conciliation procedure will be required after the second reading. Indeed, it is not yet clear whether parliament will be willing to vote on the council's common position, as it does not provide a definitive budget.

Under pressure from the French research minister, Claude Allègre, the council agreed to set up a ministerial meeting on the management and administration of FP5 at the end of April. Allègre's most radical suggestion — which has attracted little support — is that responsibility for management of the programme should be devolved from Brussels to national research organizations.

Alison Abbott

Senate throws out bill to ban cloning of human embryos

[WASHINGTON] The US Senate last week rejected a bid by Republican senators to ban the cloning of human embryos. After intense lobbying from organizations representing biomedical researchers and fertility physicians, senators refused, by a 54 to 42 vote, to bring the bill to the Senate floor for a vote.

To block the vote on 11 February, 42 Democrats were joined by 12 Republicans. The bill, written by Senators Christopher Bond (Republican, Missouri) and Bill Frist (Republican, Tennessee), the Senate's only physician, would have made the cloning of a human embryo a crime punishable by a sentence of up to ten years in prison (see *Nature* **391**, 623; 1998).

In a separate move last week, Harold Varmus, the director of the National Institutes of Health, did not appear as scheduled to testify on cloning before a meeting of the subcommittee of the House of Representatives Commerce Committee.

The Department of Health and Human Services declined to have Varmus testify because committee leaders had scheduled him to appear after Republicans, including

Frist, and religious figures. Administration officials usually testify first at congressional hearings.

France puts homeopathy under the microscope

[PARIS] The French body that regulates the practice of medicine — the Conseil de l'Ordre des Médecins — has commissioned a scientific evaluation of homeopathy. The move coincides with its adoption of a report recommending that the practice should be integrated into medical syllabuses, pointing out that people are already reimbursed for some homeopathic treatments by the French healthcare system.

More than 3,000 physicians already practise homeopathy in France. But the report criticizes the almost complete absence of formal training or university courses. "Recognized university and hospital training would be desirable," according to the conseil.

NZ researchers lobby for increase in funds

[SYDNEY] The Royal Society of New Zealand is seeking an urgent meeting with the new prime minister, Jenny Shipley, after urging the coalition government to meet a previous commitment to increase research funding to 0.8 per cent of gross domestic product by

2010, a target first announced by the former prime minister, Jim Bolger, in August 1996.

The present rate is 0.52 per cent, and there has been widespread concern that the government will 'cap' funding for research in its May budget (see *Nature* **391**, 426; 1998). In a joint statement issued on 16 February, the president of the society, Sir John Scott, and the president of the Academy Council, George Petersen, also urged the government to lift an apparent "threat" to cut back a promised increase in funding to the Marsden Fund, which supports basic research.

UK backing for nuclear dump underground

[LONDON] The British government's radioactive-waste management advisory committee insists that deep underground disposal remains the safest method of disposing of 'intermediate-level' radioactive waste from nuclear power stations. Its advice, in a report published last week, comes in the wake of the previous government's decision to suspend research into a deep repository for radioactive waste at Sellafield in northern England.

The report suggests "testing public opinion" on the idea of a single repository for intermediate-level and high-level waste, and floats the idea of an international repository shared by several countries.

Japan's nuclear agency set to be replaced

[TOKYO] The Japanese government last week approved a bill to replace its nuclear power research and development organization that has been tainted by a series of accidents and cover-ups. The bill to set up an organization to replace the Power Reactor and Nuclear Fuel Development Corporation (PNC) will be submitted to the current ordinary session of Japan's parliament.

The changes may come into effect as early as next October. The replacement of PNC is part of Prime Minister Ryutaro Hashimoto's reforms following the public outcry over a series of accidents and the subsequent compilation of false reports at PNC facilities last year.

Japanese call for human cloning moratorium

[TOKYO] Japan's science council, an advisory body to the education minister, has called for a moratorium on university research into human cloning, as well as the manipulation of cell differentiation and nuclear transfer, which was used to produce Dolly the sheep.

A report recommends that research into human cloning at universities should be regulated by guidelines instead of a legally

binding ban, to avoid stifling other research using similar technologies.

Retirement rule waived for Russian lab chiefs

[MOSCOW] Two directors of Russian scientific institutes have been allowed to stay in post despite having passed the age limit of 70 laid down by the Russian Academy of Sciences for its full members.

Guriy Marchuk, formerly president of the academy, and Ludvig Faddeev will retain their positions as directors of Moscow's Computational Mathematics Institute and the St Petersburg branch of the academy's Institute of Mathematics respectively. The decision was taken by the academy's presidium because of the absence of any competitors for these posts.

Congressman seeks funding boost for NIH

[WASHINGTON] Congressman George Gekas (Republican, Pennsylvania) introduced last week a House of Representatives resolution urging an increase in government spending on biomedical research of \$2 billion in 1999. This would boost the budget of the National Institutes of Health (NIH) by 14.7 per cent, to \$15.65 billion.

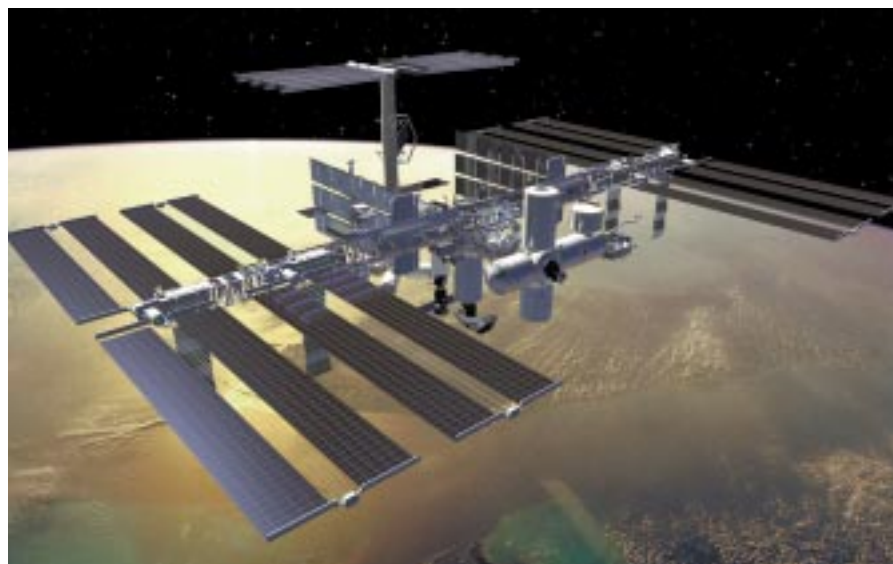
The resolution is co-sponsored by

Congressman John Porter (Republican, Illinois), who chairs the appropriations subcommittee that funds the NIH. President Bill Clinton asked Congress in his 1999 budget request to increase NIH spending by 8.4 per cent, or \$1.15 billion. A Senate resolution virtually identical to that of Gekas was introduced on 29 January by Arlen Specter (Republican, Pennsylvania), chair of the Senate subcommittee that funds the NIH.

French agency backs inquiry into lab results

[PARIS] Claude Griscelli, director-general of the French national biomedical research agency INSERM, last week lent its support for a rapid and "transparent" investigation of allegations surrounding the agency's Laboratory of Nutrition, Lipoprotein Metabolism and Atherosclerosis at the University of Rennes 1. The allegations concern research into a 'lipolysis stimulated receptor', a molecule involved in fat degradation (see *Nature* **391**, 519; 1998).

The agency's statement said it would be "inadmissible" for a national research agency to allow the laboratory to continue its activities "if the results were definitely known to be tainted by insincerity". But it argued that the affair could ultimately be resolved only if the results could be reproduced by scientific teams elsewhere.



Science struggles to gain respect on the space station

Although much has been made of the value of the international space station to science, many researchers remain deeply sceptical. But useful science may still emerge, even though various obstacles remain to be overcome.

In May 1994, Daniel Goldin, head of the US space agency NASA, invited physicist Samuel Ting of the Massachusetts Institute of Technology to brief him on an idea Goldin had heard about. Ting, it seemed, wanted to put a daring and original — if controversial — experiment on the space station that NASA and its international partners plan to launch into Earth orbit.

The Alpha Magnetic Spectrometer (AMS) would search for naturally occurring antimatter particles — anti-helium and heavier — which had long been theorized about, but never detected. By letting billions of cosmic rays pass through a large permanent magnet, Ting hoped the trajectories of one or two oddballs might bend the 'wrong' way. If his sensitive antimatter detector could score even one hit, the scientific payoff would be enormous. Goldin loved it.

Ting had never done an experiment in space before, and NASA had made no general call for proposals. Individual scientists don't usually get to pitch unsolicited ideas to

the top US space official, but then Ting is no usual scientist. A Nobel laureate and one of the physics world's high flyers, he offered Goldin a high-profile experiment at relatively low cost. The AMS will use a Chinese-made magnet bought at a bargain price.

After NASA engineers were sent scurrying to confirm that the project was feasible, Ting asked the US Department of Energy's high-energy physics division to sponsor his experiment. A DOE peer review panel endorsed the AMS, even though many physicists believe it is a long shot. In answer to criticism that the experiment will not settle the question of antimatter's existence if it fails to score a hit, Ting has broadened his objectives to include gamma ray astronomy and the search for indirect evidence of dark matter.

The energy department then agreed to find \$3 million of the AMS's price of roughly \$49 million — the rest comes from an international consortium of partners — and NASA agreed to spend \$13 million on supporting hardware, plus two free rides into space. By September 1995 the AMS was approved. It will fly on the space shuttle next May and will be among the first group of 'attached payloads' fixed to the outside of the space station in 2002, where it will remain for

three years. Now, whenever Goldin stands in front of a congressional committee or gathering of scientists to expound on the wonderful research that will be done on the station, one of the first experiments he mentions is Ting's.

The story illustrates more than just the power of a Nobel prize. It shows both the fervent hope among the space station's builders that it will produce world-class scientific results, and their desire for respect from the larger scientific community, which fears that it won't. Indeed, while many scientific societies have damned the station with faint praise, others have actively opposed it, including the American Physical Society, the European Physical Society, and the American Society of Cell Biology (ASCB).

Gaps in peer review

Some of the criticism has been quite harsh. Ursula Goodenough, a cell biologist at Washington University in St Louis and past president of the ASCB, wrote in a 1996 letter to Goldin: "The 'frontier' of microgravity never did interest first-rate scientists, physical or biological, in the first place, and this is all the more true now that it is clear that nothing of any real interest has emerged from the many in-flight studies on the effects of microgravity on this or that."

In a response to an Internet-based debate about the merits of space station science last year, Goodenough acknowledged that space experiments are peer-reviewed by scientists from outside NASA, who inevitably choose the best proposals from among the ones they are handed. But these reviewers "are never asked whether *any* of the proposals is worth the cost needed to fly them. It's assumed that [a certain percentage] will go up. Period." The problem, she says, is that "the entire concept of putting the [station] up there has not been subjected to peer review".

But this assumes that the space station is a science project, which of course it is not. It is being undertaken for many reasons, including national prestige, employment for aerospace workers in many industrialized nations, global technopolitics — particularly a desire to help prop up the Russian aerospace industry — the unwillingness of Americans to abandon an astronaut programme to which they have a deep emotional attachment and, perhaps most important, the project's own political momentum.

Dana Rohrabacher (Republican, California), a station backer who chairs NASA's oversight subcommittee in the US House of Representatives, said last year at the height of his frustration over delays and cost overruns: "I find myself admitting that, if NASA were to propose the space station today, I couldn't support it knowing what I know now." But it has long been too late to turn back.

This briefing has been written by Tony Reichhardt, with Alison Abbott in Munich and additional reporting by Asako Saegusa in Tokyo.

On other pages | [Europe rejects use of animals for research: p733](#) | [An experiment in cooperation: p734](#)

Scientific opposition to the space station is partly the result of its enormous cost. Senator Dale Bumpers (Democrat, Arkansas), who annually tries to kill station funding in Congress, complained last year that: "One shuttle flight to the space station will cost almost as much as the entire \$454 million budget of the National Institute on Aging."

The US contribution to hardware development alone is \$17.4 billion, with almost \$4 billion coming from the European Space Agency (ESA), \$2.5 billion from Japan, \$1 billion from Canada, a rough estimate of \$350 million in annual spending by Russia, plus smaller contributions from junior partners such as Brazil. Depending on how one does the accounting, the bill will run to between \$50 billion and \$100 billion, all-inclusive, to build, launch and operate the station for a decade or more in orbit. But the view that this money is being taken from space science (or from other science projects) has been generally discredited — no science managers at NASA or ESA headquarters seriously believe they would benefit if the station were cancelled.

Deduct the enormous cost of their orbit-

ing laboratory, and space-based researchers are no richer than their terrestrial colleagues. The grant money NASA gives to scientists in the field of space biomedicine is "trivial," says Jeffrey Borer, a professor of cardiovascular medicine at Cornell University. Borer does not do space-based experiments himself, but chairs a committee overseeing cooperation between NASA and the National Institutes of Health (NIH).

The same holds generally for other kinds of microgravity research. Last autumn, for example, NASA awarded 26 research teams a total of \$6 million in five-year grants related to fundamental physics in microgravity, an annual average of only \$46,000. In many cases, most of that money goes direct to aerospace companies to build expensive space hardware. If space station experimenters are accused of being pampered, they certainly don't feel as if they are.

They often do feel defensive, however, and embarrassed by press releases and advertisements promising cures for cancer and AIDS from station research. "The space life sciences community has never tried to justify the whole costs of the space station on the

basis of our science," says Alain Berthoz, a neuroscientist from the College de France in Paris. Berthoz's group has flown ten space experiments and plans to study spatial perception using virtual reality on the station. "But the station has been built, for whatever reason, and there are interesting questions which can only be answered in zero gravity."

What microgravity offers

Such questions may be few, but they are intriguing. In space, subtle fluid transport and crystallization processes, which are masked by gravity-driven convection on Earth, can be studied in detail. Muscles atrophy, bones lose mineral, fluids shift inside the body, and balance organs cease to work properly.

Some of these conditions are potentially interesting to terrestrial researchers. Examples of experiments that microgravity researchers say could be significant to the general scientific community are:

- *Continuing study of tree-like structures called dendrites in solidifying metals.* Free from the dominating influence of gravity, the dynamics of dendrite formation can be observed on very short time scales (using high-frame-rate video expected to be available on the space station) while experimenters manipulate such variables as temperature and hydrostatic pressure. The work should improve theoretical models of dendrite formation, a key factor in determining the strength and durability of metal alloys.

- *Investigation of the cellular mechanisms behind bone deterioration.* Astronauts lose about one per cent of their bone mass for every month they stay in orbit. Bone deterioration due to spaceflight may be linked to progressive bone diseases such as osteoporosis, but it happens at a much faster rate. Is there a common mechanism? On the space station, researchers could evaluate how different levels of mechanical stress (starting with its near complete absence in weightlessness) affect gene expression in osteoblast cells that promote bone growth. For the first time, scientists will be able to study bone modelling and remodelling in multiple generations of animals that have never experienced gravity.

- *ACES (Atomic Clock Ensemble in Space).* A French-led experiment scheduled to be attached to the outside of the station beginning in 2002, ACES will use microgravity conditions to improve by a factor of ten the accuracy of a laser-cooled caesium atomic clock (which uses the same laser-cooling techniques that won last year's Nobel Prize in physics).

On earth, the atomic clock measures the fundamental oscillation frequencies of caesium atoms during their trajectory in an 'atomic fountain' contained within a one-metre vessel. If the atoms in the fountain are not held back by gravity, they can be launched

No animals allowed for European researchers

European life scientists conducting research on the space station may have to work with a severe handicap compared with their international colleagues: they may not be allowed to use laboratory animals.

Although experiments organized through the European Space Agency (ESA) are paid for by individual member states, the agency itself administers the research programme. And because Germany, the biggest European contributor to the station, is refusing to support the use of animals for experiments in space, ESA has imposed an unwritten rule: no rats, no frogs, no animals of any kind.

"This is a great pity for our [research] community," says Alain Berthoz, a neuroscientist from the College de France in Paris and a veteran space experimenter. One key topic for space biology, he says, is the influence of gravity on neurological development. And that topic, he says, can only be addressed by flying living animals in space.

Didier Schmitt, director of ESA's life sciences department, considers the implications of the moratorium sufficiently problematic to have formed a small task force to consider the issue, and hopes to have it lifted as a general principle. "If we don't do [animal experiments], we will not be able to catch up with the United States and Japan," he says. He wants to convince Germany that scientists from other ESA states should not



Banned: unlike the early 1960s (above), primates can no longer fly in the US.

be prevented from carrying out animal experiments — even if Germany chooses not to fund such experiments itself.

European scientists have flown animals on past space missions through agreements with either the United States or Russia. Now that

all ESA partners have to agree on rules for the use of ESA facilities on the space station, the issue is more complicated. Schmitt fears that European researchers may react to the moratorium by reaching bilateral agreements with the United States, Russia, or Japan, and that the coherence of ESA's research could suffer.

US researchers, meanwhile, will have to operate under separate restrictions. For political reasons, no non-human primates will be allowed on the space station. And that, say life scientists, will rob them of an important research tool, particularly for cardiovascular studies.

at a lower velocity, and so can be observed for a longer time. Accuracy is inversely proportional to time of observations.

These, however, may be exceptions that prove the rule. There remains broad agreement that much of the research planned for the station is likely to remain tangential to the central concerns of biomedicine and materials science. Its greatest impact will be felt in the small community already studying problems related to spaceflight — a vital research area only if we assume that increasing numbers of people will someday travel,

or even live, outside of normal Earth gravity. Plant studies are likely to focus mostly on growing crops for astronaut crews. Combustion research in microgravity, while revealing some new information about the basic physics of flame propagation or turbulence, will primarily influence those who write fire safety regulations for spacecraft.

Lothar Willmitzer, director of the Max Planck Institute for molecular plant physiology in Potsdam, illustrates the difficulty space experiments have in competing with ground-based research on a cost-benefit

basis. Work on gravitropism, the directional growth of plant tips in response to gravity, is interesting basic biology, he says, and space experiments in this area have been well designed. At the same time, mutants of the standard reference plant *Arabidopsis thaliana* have been developed that have aberrant responses to gravity, causing their roots to grow in all directions. Using mutants to explore the genetic mechanisms behind the gravity response is much cheaper than rocketing an experiment into Earth orbit.

Still, the quality of space experiments is

Bartering for experimental time and space has already begun

The space station is proving to be a challenging experiment in international collaboration as well as being a hugely complex engineering job. "In effect, an international space agency has been created for the station," says John Logsdon, a space policy analyst at George Washington University in Washington. He points out that a 300-person multinational management group is already planning for the station's continuous use and operation.

Representatives of the 15 major station partners — the United States, Russia, Japan, Canada and 11 members of the European Space Agency (ESA) — gathered in Washington last month to approve formally the sharing of facilities on the station, from laboratory space to electrical power.

NASA, the biggest financial contributor and overall project manager, will have almost full use of its own laboratory module, plus slightly less than half of the Japanese and European research laboratories. ESA and Japan are each allocated 51 per cent of their own laboratories, and Canada 2.3 per cent of all three research modules in exchange for building the station's robot arm.

Resources such as storage space, power, and crew time also will be shared, with NASA receiving 77 per cent, Japan 13 per cent, ESA 8 per cent, and Canada 2 per cent.

These formulas apply only to the non-Russian part of the station. The project's second biggest partner in terms of hardware contributions will retain full control of its own resources, as well as of the two research modules it plans to add in 2002. Crew time will be evenly divided, with half going to Russia and half to the rest of the partners. For all practical purposes, says Logsdon, "the station is divided in two".

These, however, are only the opening positions. Bartering of time, hardware, and laboratory space has already begun before the first element is even launched. ESA, lacking any place of its own to attach exterior payloads during the early years of assembly, is building a pointing device for astronomical and Earth-observing instruments in exchange for some of NASA's



Family portrait: officials from the 15 countries that will share in running the space station.

'turf' on the outside of the station.

Brazil last year signed a bilateral agreement to provide a high-quality optical window in the 'floor' of the US laboratory, which it will trade for Earth-viewing time that had been reserved for NASA. And in a move that has upset many US scientists, Japan is to build a critical centrifuge for biological studies — which NASA had been planning to supply — to offset costs associated with using the space shuttle to deliver its laboratory module to the station in 2001 (see *Nature* 383, 8; 1996).

US scientists, fearing their interests were playing second fiddle to NASA's eagerness to defer any near-term cost of building research equipment, have asked to be included in any further discussions of bartering between NASA station programme managers and the international partners.

The so-called 'attached payloads' illustrate how a 'real estate market' may develop among the international partners. The NASA-owned supporting "trusses" and the Japanese laboratory both have room for mounting external payloads that have no need of pressurized conditions.

NASA has, so far, been slow to identify experiments that might occupy these prized slots. In contrast, ESA was surprised last year

to receive more than 100 responses to its call for proposals for attached payloads, which included experiments in technology, Earth observation and radiation biology.

Because of the popularity of the outside slots, ESA has decided to add external pallets to its laboratory module which is scheduled for launch in 2002. Until then, European scientists will have to queue up for whatever room ESA can barter from NASA and Japan.

The first steps towards creating a unified research programme for the station have also been taken by the international partners. In 1995, after three years of negotiation, life scientists from all the relevant space agencies, including those of France, Germany, Canada, and Japan as well as NASA and ESA, agreed in principle to share all their facilities and resources and to allocate them to projects jointly assessed as the most scientifically important, regardless of geographical origin.

To this end, the International Space Life Sciences Working Group was set up. It organizes a global peer-review exercise through 14 subdiscipline-related peer-review panels. Results from the first such joint review last year surprised nearly everyone: two-thirds of the selected experiments were from outside the United States, with more than half coming from Europe.

The lopsided result poses a dilemma. European and other non-US proposals may have ranked higher, but their funding agencies — which will make the final determination of what goes on the space station — are poorer than NASA, which also owns the lion's share of station resources. Some US experiments may therefore end up flying while non-US experiments that scored higher in this first attempt at global peer review wait on the ground.

Still to be determined, too, is how active Russia's own research programme will be. The Russian space agency already has identified more than 250 investigations that could go up on the station, but money to pay for them is in short supply. Without outside support, Russia may become a laboratory owner with no researchers of its own.

likely to improve in the space station era. According to many outside scientists, the space life sciences, once criticized for a lack of experimental rigour, have undergone a quiet transformation in recent years. NASA has strengthened its ties with the NIH, signing cooperative research agreements with 22 of its 24 institutes in subjects ranging from aging to studies of the vestibular system. While some of the motive has been political, the NIH collaboration has had real benefit, says Borer, in "bringing NASA into the mainstream", and in attracting new people with new ideas, if only temporarily, to the field.

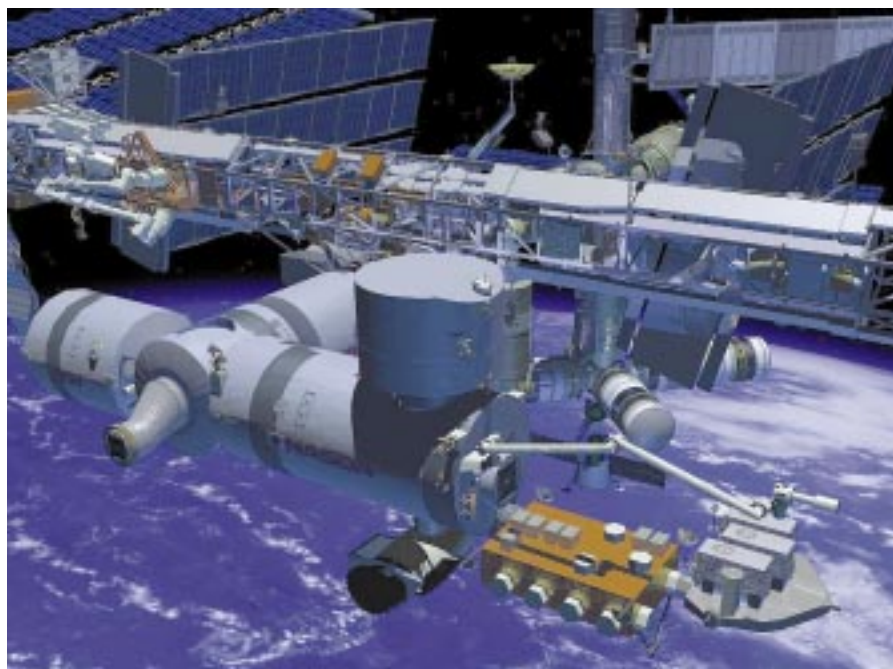
This spring's international Neurolab space shuttle mission, many of whose neurobiology experiments are being done in collaboration with NIH-funded researchers, represents "probably the best level of science" that NASA has flown, according to Kenneth Baldwin of the University of California at Irvine, a Neurolab investigator and chair of the space agency's advisory subcommittee for life sciences.

Europe has been slower to broaden its space research community. Some 95 percent of recent grant applications for ESA microgravity research, for example, were from groups that already had space experience. One reason, says Didier Schmitt, director of the agency's life sciences department, is a lack of awareness among the general scientific community about space station opportunities. "It is hard to introduce new people before even the first [station] components have been launched," he says. Schmitt hopes to help remedy the situation by recruiting 'topical teams' from the larger community to set scientific agendas for the station in different fields of research.

Alberto Passerone, chairman of ESA's microgravity working group and director of the CNR Institute for the Physical Chemistry of Materials in Genoa, Italy, says that now is the time to encourage new blood. "I am a bit disappointed in the small number of new groups taking part in approved experiments," he says.

It is possible, of course, that some discovery on the space station will turn out to be of lasting scientific importance. Researchers planning to use the station make no promises, but say they'll never know unless given the chance to try. For the past three decades they have made do with sporadic rides into space, with years passing between an experiment and the opportunity to repeat it.

Only a handful of Spacelab missions have flown, and those have lasted only two weeks. Russia's Mir has only modest laboratory equipment, which has limited what US guest investigators can accomplish. Because of these constraints, space biology has produced mostly "tantalizing suggestions rather than solid evidence," says NASA's chief life scientist, Joan Vernikos. "We haven't had the keys to the laboratory," says Arnauld Nicogossian, who heads the space agency's office



Locked in: the Japanese module (foreground) includes a platform on which experiments and observations in an exposed environment will be carried out. Behind it can be seen the Russian module, based on Mir; to the left and in the middle, respectively, are the European and US facilities.

of life and microgravity sciences.

The space station should give them, if not the keys, at least frequent access to a continuously operating laboratory. For the first time, scientists hope to be able to repeat experiments quickly and often, to study multiple generations of animals and plants in orbit, and to get statistically significant results without waiting a decade.

That, at least, is the promise. But victory is by no means assured. Many researchers admit they worry that the station will not live up to its potential.

Worry no. 1: Time

According to NASA's original plan, the space station was supposed to have been completed four years ago. The agency therefore began phasing down its Spacelab programme, the main venue for space-based research, which now has only one mission left — Neurolab.

The station schedule has slipped badly, so that construction will not even begin until this summer, and the first research 'utilization flight' is not until January 2000. Full scientific use will not be achieved until 2003, when a full crew of seven is onboard to conduct experiments. Many space biologists worry that their field will shrivel and die as researchers grow tired of waiting for data.

"We've lost a lot of people already," says Laurence Young of the Massachusetts Institute of Technology, a veteran space experimenter who heads NASA's new Space Biomedical Research Institute (SBRI). Without data from space, "intelligent people will find other things to do with their lives".

The space research community has

pleaded with NASA to add more shuttle research flights while the station is being built. So far the answer has been mixed. One such mission has been added to the flight schedule for next October and another is planned tentatively for late 2000. But space will be limited, and part of the flights will be reserved for commercial payloads.

Meanwhile, life scientists say NASA is woefully behind in funding preliminary work on space station experiments, rather than simply generic concepts. "We're at the point where we have to start doing the groundwork, or [the experiments] aren't going to be ready for flight," says Martin Fettman, a Colorado State University veterinarian and pathologist who flew on a Spacelab mission as a scientist/astronaut in 1993, and who chairs the outside advisory panel for the station's centrifuge facility.

Worry no. 2: Facilities

As space station managers have run into budget problems, they have borrowed funds meant for research equipment to pay for the station itself. As a result, some key facilities have been delayed — including the 2.5-metre-diameter animal and plant centrifuge that will allow life scientists for the first time to control different levels of gravity. Young echoes the feelings of many space biologists when he frets: "We'll build a house, but not have any furniture."

Another concern is the station's ability to deliver power, communications and data processing to experimenters, and to upgrade those capabilities over time. Real-time, high-resolution video data downlinked to the ground will be "essential" for microgravity

researchers, says Martin Glicksman, a materials scientist at Rensselaer Polytechnic Institute who has pioneered 'telescience' during Spacelab missions. But continuous high-bandwidth communications is required.

NASA promises that high-capacity data transmission will be available by 2002 (both Russian and US data relay satellites are planned), and talks about switching to commercial satellite networks to supply greater capacity in the future. In a cautionary move, Japan and Europe reserve the right to provide their own data relay systems.

Perhaps the greatest worry among microgravity researchers is not knowing how free of vibration the station will turn out to be — a critical question for delicate fluid physics and crystal growth studies. Researchers have been promised that acceleration forces won't interfere with their work. But equipment designed to damp out vibrations from myriad sources on the station — whirling fans, motors, astronauts exercising on treadmills — worked only partially on its space shuttle test flight and may not be tested again before being installed in experiment racks in NASA's lab module in 2000.

US investigators can only hope that this Active Rack Isolation System — and other untested hardware — works, or that NASA has enough money to fix it. European and Japanese researchers may go with an alternative Canadian design for damping out vibration. Japan's space agency NASDA has been especially concerned that the station may not be as "quiet" as advertised.

Worry no. 3: Money

European users cannot be certain how many space station experiments their sponsoring governments will be able to afford. The two main ESA players in the project, Germany and France, are already having difficulty finding 'utilization' funds.

The German-led Fire Detection Infrared Sensor System was one of six experiments selected by an ESA review board for a coveted place on the station's exterior. But the experiment has been rejected for funding by the German space agency DLR, and the principal investigator has been asked to submit a scaled-down version. The DLR has had funding for German (as opposed to ESA) space research cut by 21 per cent over the past

four years, with microgravity science bearing a disproportionate reduction.

The situation in France is even more drastic. One of the first acts of the new research minister, Claude Allègre, last October was to cut the 1998 budget for microgravity research to zero, as part of his bid to find more money for research posts. He has since restored some of the money, but the final amount is in question. Only Italy has reallocated money to pay for station experiments. But Giovanni Big-nami, science director for the Italian space agency ASI, admits that this will strain funding for other space activities in coming years.

Worry no. 4: Manpower

Astronauts will have only limited time to run science experiments during the five years of station assembly. Ironically, the situation will get worse as more research equipment arrives, because the crew will have more assembly tasks and spacewalks to do. John-David Bartoe, research manager for the station at NASA's Johnson Space Center in Houston, estimates that the total time the initial crew of three US astronauts and Russian cosmonauts will devote to research will

Commercial labs to rent: good views, priority booking

Government officials on both sides of the Atlantic are optimistic that private industry will pay to use the space station for its own research projects. And a generous portion of onboard resources — laboratory space, electrical power, crew time, and the like — has been set aside for this purpose; now all that is needed is customers.

European station resources reserved for industrial use are expected to amount to between 20 and 30 per cent. The US reserve will be between 30 and 40 per cent — compared with 30 percent each for basic life science and microgravity research. NASA chief Dan Goldin would like to see this proportion climb eventually to 60 per cent.

German research minister Jürgen Rüttgers goes one better than Goldin — he would like commercial users to pay as much as possible for the station. And Claude Allègre, the French research minister who has complained loudly about the costs of station use, would be happy to see no government-funded basic research at all, letting industry take all the laboratory space.

This kind of talk may warm the heart of politicians who hope the private sector will help foot the station's enormous bill. But it frightens government-funded academic researchers, who expect to be rudely bumped whenever a paying customer comes along.

Perhaps they have no need to worry. Despite years of government efforts to enlist commercial partners, industry has so far shown little interest in spending real money on space experiments. NASA alone has spent

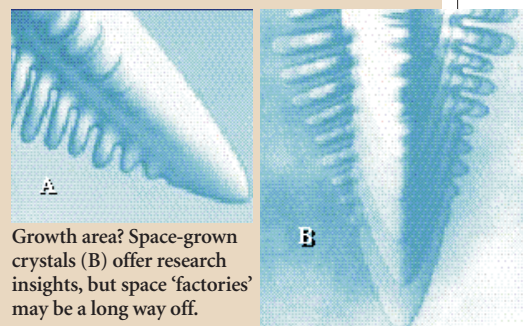
about \$40 million a year for the past decade trying to prime the pump of private space investment primarily through a network of Commercial Space Centers (CSC) affiliated with universities or private companies, which offer free rides into space for CSC-sponsored research.

As of last year, NASA could claim some 150 private US firms as partners in this commercial space network. But their investments have mostly been limited to small, in-kind payments, such as donating a researcher's time and laboratory facilities, or contributing a few tissue samples or crystals to be launched into orbit.

Some projects flown on the space shuttle by 'name' companies like Merck and Genentech have been "advertising, let's face it", says one senior NASA science official. In an advisory committee meeting last year, Edward Gabris, who oversees commercial payloads for NASA's office of microgravity and life sciences, admitted that "nobody beats a path to my door" to send privately funded experiments into orbit.

Hartmut Ripken, director of space station activities at the German space agency DLR, says European industry's lack of interest is understandable, given the past lack of flight opportunities on the space shuttle, and the inadequate facilities for industrial research on Mir. The space station will be different, he believes, and will draw private sector interest once the research results start coming back.

The bad news for would-be space entrepreneurs is that any commercial



Growth area? Space-grown crystals (B) offer research insights, but space 'factories' may be a long way off.

operation in orbit has to compete with far cheaper ground-based alternatives. Commercial protein crystal growth (PCG) in space is often touted as a likely money-maker. Crystals tend to grow larger in microgravity, yielding more suitable material for X-ray diffraction analysis.

Pharmaceutical companies will pay good money for such crystals, say advocates of PCG, because it will help them design more effective drugs. But even on the ground, protein structure analysis is no longer a preferred method of drug design, which currently focuses more on high-throughput testing of candidates generated through combinatorial chemistry and gene libraries.

"Commercial payloads are getting far more space than is justified," complains one materials scientist, who questions whether a space manufacturing boom will ever materialize when the cost of reaching orbit is so high. He quips: "If Rumpelstiltskin took straw into space and spun it into gold, he'd still lose money."

drop from 50 hours a week early on to 15 hours by the time the station is able to house its full complement of seven people in 2003.

Space researchers design their experiments to run as autonomously as possible, because crew time is the scarcest commodity in orbit. But initially the station may seem like a step backward from Spacelab missions, where scientist 'payload specialists' were aboard just to do research (including almost all the Europeans, Japanese and Canadians who have flown to date). "I think people will be disappointed" in how little time the astronauts have for research as they build and troubleshoot their new home in orbit, says one veteran space experimenter. The research community has waged, and so far lost, a battle to have a payload specialist on the station exclusively for science.

John Blaha, a former NASA astronaut who spent four months on the Russian Mir space station in 1996, earns praise from scientists as a dedicated experiment operator who worked hard to deliver as much data as possible in orbit. Now an insurance executive in Texas, Blaha insists that "NASA needs to shift its culture" if it wants astronauts to do quality research on the space station.

Micromanaging the crew will not work on flights lasting several months, he says. Astronaut/experimenters should be well versed in the goals of a research project, but should then be left to operate an experiment on their own schedule. They also need to be able to talk direct to investigators on the ground when they run into a problem. If these guidelines aren't followed on the station, Blaha says flatly, "we won't be as efficient and we won't get as much done".

Will these and other warnings from scientists be heeded? The space research community, long accustomed to being second-class citizens in the world of 'piloted' spaceflight, can only hope for the best. One of the many ironies of the space station is that this most elaborate home ever built for people in space will be a less-than-ideal platform for doing the basic research needed to be able to send humans safely to Mars — ultimately its most convincing rationale.

The one piece of equipment SBRI director Laurence Young says he would want most for such research, a centrifuge big enough for humans, was thrown out long ago as too expensive. The station will contribute little knowledge about the threat from space radiation — some say the biggest biological hurdle facing Mars crews — because its orbit is protected from the most dangerous particles.

But the space station is a compromise, and no one will come away entirely happy — not the researchers, not the engineers working under great fiscal and institutional pressure, and not the politicians. They will get a station far less grand and more expensive than the one Ronald Reagan promised the world back in 1984. □

When astronauts refuse to volunteer

On next April's Neurolab space shuttle mission, four scientist/astronauts have agreed voluntarily to insert tungsten needle electrodes into their legs to monitor nerve activity — all in the name of science.

Not all astronauts are so obliging. US crew members on the Russian Mir space station have in the past refused to perform even simple, non-invasive tasks simply because they didn't want to. It doesn't matter that the research has been peer reviewed and paid for, or that a scientist may have spent months, or even years, planning a test. By the rules of medical ethics and informed consent, an astronaut/test subject is free to just say no, and not give a reason.

For scientists who conduct human research in space, astronaut cooperation has become a troubling and sensitive issue, which they are loath to discuss on the record. "This is not just a theoretical problem," says one researcher who has had tests refused. It has happened more than once on Mir.

On Earth, the scientist would simply get another volunteer. In space, only six test subjects might be available for an entire research project. When an astronaut balks at having his or her blood drawn, there is no-one else to turn to, and the experiment is likely to be scrapped.

Most astronauts are willing participants in research, and many investigators — particularly those who have worked with 'payload specialists' flown specifically to conduct science — have only good experiences to report. But some astronauts take the research less seriously.

Scientists who need critical biomedical data from immediately before and after a spaceflight to compare with in-orbit results report that sometimes the measurements get done and sometimes they don't, depending on the individual astronauts.

Russian cosmonauts on Mir have presented a different problem — likely to crop up again with the space station — by demanding to be paid as test subjects. NASA has refused, arguing that its astronauts get no such bonuses. The data have gone uncollected.

Since the first crews on the space station will alternate between two Russians with one American and two Americans with one Russian, "a lot of anxiety is building up in the research community" over the availability of test subjects, says one scientist.

Career NASA astronauts have in the past been wary of scientists treating them as human guinea pigs. It can be more than just a matter of pride or privacy — the discovery of an unexpected cardiac arrhythmia or



Testing time: not all astronauts participate happily in research.

susceptibility to space sickness can ground astronauts or otherwise damage their careers. This has contributed to another tension, between academic biomedical researchers and the

doctors at NASA's Johnson Space Center in Houston who are responsible for crew health and safety. Outsiders complain that the NASA doctors have received preferential treatment in flying 'experiments' that aren't as rigorously peer reviewed.

Communication between the two camps is not always good. More than once, according to several sources, an outside researcher running a carefully controlled experiment in human physiological responses to spaceflight has had an experiment ruined or compromised after NASA doctors, in private conference with an astronaut/test subject, prescribed medicine (for example, for space sickness) without informing the researcher.

Discussion of these issues has reached a high as NASA administrator Dan Goldin, and several internal NASA committees and outside working groups have met to work out a compromise between astronauts' concerns about privacy and informed consent, and the needs of biomedical researchers. Since standards for medical ethics may vary among the space station's international partners, a Human Research Multilateral Review Board will also be established to set consensus guidelines for human research.

Ronald Merrell, the head of Yale University's department of surgery and chairman of NASA's advisory group for space flight medicine, believes the problems can be resolved, particularly if astronauts are treated as informed partners in conducting research. "You make it their project," he says.

Laurence Young, a researcher at the Massachusetts Institute of Technology who heads NASA's Space Biomedical Research Institute, agrees. Still, he says, space crews "have a privilege but also a responsibility", and part of their responsibility includes helping scientists to answer fundamental research questions that the station will be built, in part, to address.

Portuguese science needs a shake-up

Sir — Adelino V. M. Canário calls for a dramatic change in the way Portuguese science is funded and managed (*Nature* **390**, 656; 1997). There is certainly a need for change, but it should be less dramatic, better organized and not based on fallacious assumptions.

His graph of gross domestic product versus number of publications in European countries is meaningless on its own. It brings to mind the study showing that high expenditure on soap in hospitals correlates very significantly with the number of deaths, which does not mean that soap consumption contributes to mortality.

Similarly, it must be remembered that 24 years ago, and after almost half a century of dictatorship, Portugal, with its 25% illiteracy level, matched standards in developing countries while in Ireland there was no illiteracy. Illiteracy has now been almost eradicated in Portugal and about 32% of secondary school students enrol in universities. So, within one generation, a revolution has occurred within the public education and research sector.

At present, these changes need to prove their usefulness for a society which is asked to finance science in the universities. It is true that academic institutions suffer from chronically low funding. However, it is also true that the lack of direct evaluation of

academic staff (by students) is responsible for the frequently low quality of teaching in universities. As a result, individuals with a poor teaching record may easily reach senior tenure positions within the academic hierarchy.

Furthermore, progress in an academic career is often unrelated to scientific productivity and more or less guaranteed by a law ensuring lifelong teaching contracts, which provide little incentive to publish.

There are several bottlenecks in planning and managing research activities, but they have nothing to do with any excessive weight that undergraduate students may have in electing governing bodies at the universities. Canário confuses the issues.

In our opinion, these obstacles are mostly due to:

- lengthy evaluation of research and development project proposals at a national level (up to two years);
- delays in transferring funds to the universities for execution of projects (up to one year);
- anachronistic administrative rules and an acute shortage of skilled administrative officers capable of handling the administration of research projects;
- overburdening of researchers with minor administrative duties (often as much as 80%

of useful time), which transforms them into amateurish accountants.

So, before more funds are made available, as Canário asks, the administration of research at universities should be fundamentally restructured so that scientists are held accountable for the scientific results they produce rather than for their book-keeping. A strengthening of research units should provide them with the necessary basic equipment and should not be transformed into long-term funding which is quickly taken for granted. The competition for funding of research ideas between research centres is a sound system which has worked well in the past and should not be changed.

Beyond the research goals set by the European Commission in Brussels, new scientific programmes should take into greater consideration the priorities of both the national public and private sectors and include, in the case of Portugal, the once blooming but now almost abandoned research sector in Africa.

Tomasz Boski

Helena D. Galva

UCTRA — Universidade do Algarve,
Campus de Gambelas,
8000 Faro,
Portugal
e-mail: tboski@si.ualg.pt

Celebrated errors

Sir — J. L. Heilbron and W. F. Bynum say, in their Commentary article about anniversaries of note that occur this year, that Stanley H. Cohen (Nobel prizewinner, 1986) and Herbert W. Bayer (*sic*) opened up the field of genetic engineering (*Nature* **391**, 13–16; 1998).

The Stanley (no-initial) Cohen who received the Nobel prize for the discovery of epidermal growth factor is not the same as Stanley N. Cohen who laid the foundations of recombinant DNA technology along with Herbert Boyer (not Bayer), both of whom, in my view, also merit the prize.

Charles Weissmann

Institute for Molecular Biology,
Dept I, University of Zurich,
Hoenggerberg,
CH-8093 Zurich,
Switzerland
e-mail: weissma@molbio1.unizh.ch

Sir — J. L. Heilbron and W. F. Bynum mention that the US National Aeronautics and Space Administration (NASA) made history by putting into orbit three space stations called sky labs. In fact, Skylab was a

single space station, launched unmanned, all up on 14 May 1973, and was visited by three successive three-man crews, the last of which departed on 8 February 1974.

On 11 July 1979, Skylab struck the Earth's surface, scattering debris from the southeastern Indian Ocean to Western Australia. Readers interested in Skylab can visit the NASA Web site (<http://www.ksc.nasa.gov/history/skylab/skylab.html>).

Julian A. Hiscox

Division of Molecular Biology,
IAH Compton Laboratory,
Compton, Berks RG20 7NN, UK
e-mail: julian.hiscox@bbsrc.ac.uk

Biology versus physics

Sir — Your leading article “Biology versus physics” questions the existence of Kuhnian paradigms in biology (*Nature* **391**, 107; 1998). I would offer as a candidate the demonstration by Lynn Margulis and others that mitochondria are endosymbionts descended from formerly free-living proteobacteria. For, to those of us interested in both evolution and religion, the confirmation that human and other

eukaryotic cells were really bacterial cooperatives (in Whose image?) certainly felt like a double-strength paradigm shift.

Arndt von Hippel

1649 Bannister Drive,
Anchorage, Alaska 99508, USA
e-mail: 102251.527@compuserve.com

‘Mad’ science wanted

Sir — Towards the end of your interview with Daedalus, he points out that even Sir Peter Medawar felt that 80% of his research activity had been completely wasted, and that this is a typical record (*Nature* **390**, 126–127; 1997). Perhaps his main point was that a 20%-efficient activity shouldn't be taken too seriously. But can Daedalus suggest an entropy-defying device by which I can determine which of my activities belong in the 80% ‘fruitless’ category — and trade those for exploring not-so-serious, unconventional ‘mad’ experiments?

Boryeu Mao

Pharmacia and Upjohn Inc.,
Kalamazoo, Michigan 49001, USA
e-mail: bmao@raven.pnu.com

Fixed hotspots gone with the wind

Ulrich Christensen

Island chains such as Hawaii are produced by hotspots – points of volcanic activity driven by plumes of hot rock. New analyses reveal that hotspots are much more mobile than had been thought.

Ever since we have known that the Earth's tectonic plates are in relative motion, the search has been on for fixed points to which absolute plate motion can be referred. For a while it was assumed that hotspots, long-lived centres of volcanic activity, provide such a reference frame. Hotspots are commonly explained by mantle plumes, narrow conduits of upwelling hot rock that probably originate near the core–mantle boundary and rise close to the surface. But it seems unlikely that plumes can be firmly anchored in a convecting mantle. Writing in *Geophysical Journal International*¹, Steinberger and O'Connell now describe how they have used kinematic models to tackle the issue — they show how plumes have swayed, drifted and twisted in the large-scale mantle flow, yet have produced the observed hotspot tracks at the Earth's surface.

The chain of islands and seamounts originating at the Hawaiian hotspot is the best example of such a track. Radiometric dating shows that the rocks become progressively older with increasing distance from Hawaii. The present distances and ages reveal how the Pacific plate has moved, with respect to the hotspot, during the past 80 million years (Myr). For example, the sharp bend at the link between the Hawaiian chain and the Emperor seamount chain (Fig. 1) is thought to have resulted from an abrupt change, at 43 Myr, from northerly plate drift to the current, more westerly, motion.

In 1971, Morgan² recognized that the various hotspot tracks on the Pacific plate can be explained by assuming that the hotspots are fixed relative to one another, and he introduced the concept of deeply anchored mantle plumes. It was later shown, however, that the Pacific hotspots move relative to those in the Atlantic³ at rates of 1–2 cm yr⁻¹. This is less than the speed of fast-moving plates (10 cm yr⁻¹), but enough to make the hotspot frame of reference suspect.

Results from seismic tomography⁴, a technique for imaging the Earth's internal structure using waves generated by earthquakes, imply that much of the return convective flow that balances plate motion occurs in the lower mantle, which therefore cannot be the immobile groundwork through which fixed plumes rise. In a first

step, Steinberger and O'Connell¹ calculate a global mantle-circulation model for the past 68 Myr, using buoyancy forces inferred from tomographic anomalies and taking the present and past plate motions as boundary conditions. In the second step, they insert plume conduits and calculate how these conduits are advected by the 'mantle wind' (a process comparable to the advection of a trail of smoke in the atmosphere). The tendency of tilted plume conduits to straighten out because of their own buoyancy is also accounted for. Strictly speaking, plumes and the large-scale circulation are parts of a single convective system. But because plume conduits are comparatively narrow (50–100 km across), they can probably be considered as separate entities that do not affect the global circulation.

In Steinberger and O'Connell's model, the plume conduits are twisted and substantially tilted from the vertical (it is assumed that plumes break up and become extinct when the tilt gets larger than 60°). The model hotspots migrate at a rate of about 1 cm yr⁻¹ in a reference frame of zero mean plate drift. However, all the Pacific hotspots move more or less consistently towards the south-east. After adjusting the drift of the Pacific plate,

the model explains the observed hotspot tracks equally well as the assumption of fixed plumes does. Because the mantle wind blows differently in other parts of the world, hotspots in the Atlantic and Indian Oceans are predicted to migrate independently from those in Pacific, in agreement with observations.

Plume advection is sensitive to the variation of mantle viscosity with depth. Steinberger and O'Connell's results are consistent with observed hotspot tracks only for a comparatively low viscosity of 1.5×10^{20} pascal seconds (Pa s) between 100 km and 400 km depth, and a high viscosity of the order of 10^{23} Pa s below 1,500 km. If the lower mantle is less viscous than that, hotspots should wander around at a higher rate than is compatible with surface observations. The low viscosity at shallow depth is required to explain the sharpness of the bend in the Hawaii–Emperor chain. In a model with a stiffer upper mantle, the plume conduit reacts to the sudden change of plate drift direction by performing a wide swing, which would result in a smoothly curved hotspot-track.

Although the idea of a viscosity increase with depth is not new, its magnitude has been disputed. A modest rise, less than an order of magnitude, had been inferred from the uplift of the Canadian land surface in response to the removal of the glacial load at the end of the last Ice Age. In contrast, the interpretation of long-wavelength anomalies of the Earth's gravity field related to subducted plates favours a stronger increase of viscosity with depth. This conclusion is now reinforced by the modelling of plume migration.

The new results put some long-standing ideas about plume migration on a more quantitative basis. What are the conse-

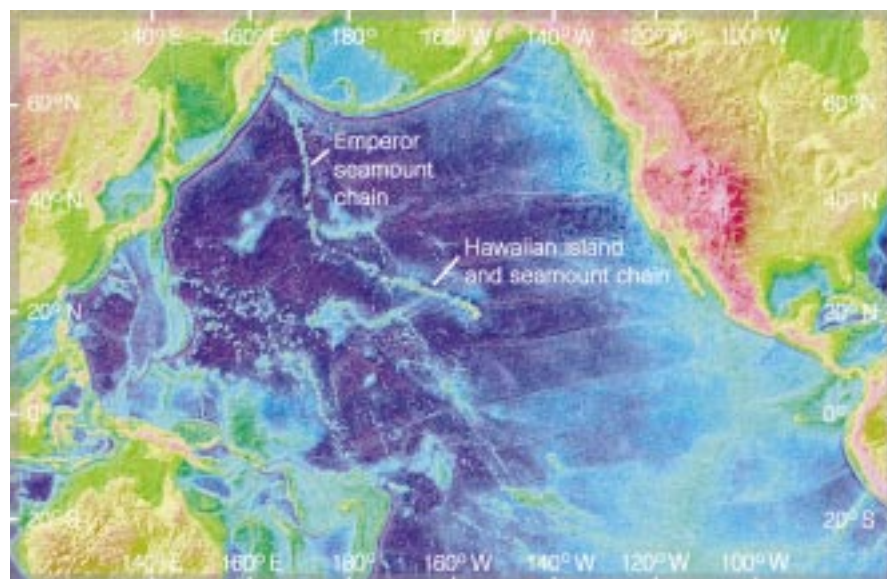


Figure 1 Topography of the northern Pacific seafloor, showing the chain of islands and seamounts originating at Hawaii, and the Emperor seamount chain. The sharp kink between the two is customarily taken to reflect a sudden change in the direction of plate motion about 43 million years ago. (Map reproduced from ref. 9, courtesy of Walter H. F. Smith.)

quences? It seems that we must abandon the convenient concept of fixed hotspots as reference points for past plate motions. Ironically, this jeopardizes some lines of argument in Steinberger and O'Connell's work. The only good evidence that we have for an abrupt change in the motion of the Pacific plate 43 Myr ago is the kink in the Hawaiian hotspot track. No other major tectonic event occurred at that time⁵ and geodynamic models based on buoyancy forces derived from subduction history, which do a good job of explaining present-day plate motions, fail to predict the change⁶. Could it be that the hotspot, rather than the plate, suddenly changed its state of motion?

To decide on this question, we need a reference point that is independent of plates and hotspots. The Earth's magnetic dipole axis, which wanders around but coincides with the rotation pole when averaged over some thousand years, provides just such a reference. The direction of magnetization acquired when a volcanic rock is cooled below its Curie temperature, the temperature at which magnetization becomes fixed, is often preserved over geological time, allowing determination of the rock's palaeolatitude — that is, its latitude at the time that the magnetic field was frozen in.

In another paper, published last year, Tarduno and Cottrell⁷ presented palaeomagnetic data for a 81-Myr-old seamount near the northern end of the Emperor chain, which indicate that it was formed at a latitude of 36°N. Another seamount further south in the Emperor chain was created 16 Myr later at 27°N. If the Hawaiian hotspot had remained fixed with respect to the rotation axis, both should have formed at 19°N, the present latitude of Hawaii. The latitude change could be explained by a movement of the entire Earth relative to the rotation axis, called true polar wander. But although this phenomenon is indeed thought to occur (driven by slight changes in the Earth's moment of inertia), its path, derived from independent data⁸, does not explain the rapid latitude shift of the Hawaiian hotspot in the time between the formation of the two seamounts.

Setting true polar wander aside, the palaeomagnetic data are consistent with a southward drift of the hotspot at a rate of at least 3 cm yr⁻¹ before 43 Myr, much faster than Steinberger and O'Connell's model predicts, and little drift after 43 Myr. These data therefore challenge the usual interpretation, also adopted by Steinberger and O'Connell for calculating their time-dependent circulation model, that the kink in the hotspot track reflects a change of plate motion. Of course, if there is no obvious reason why the motion of the Pacific plate should change, a sudden stop of hotspot migration is equally enigmatic.

Although two data points may not be

enough to make a strong case, and the possibility of true polar wander complicates the interpretation, Tarduno and Cottrell show that the way to disentangle plate drift and hotspot migration is to study the magnetization of volcanic rocks along a hotspot track in some detail. Palaeomagnetic data are abundant for the continents, but most hotspot tracks lie in the oceans and can only be sampled by ocean drilling. So the difficulties in obtaining reliable magnetization directions are far greater for seamounts than for land-based rocks. But it seems well worthwhile for palaeomagnetists to take up the challenge. □

Ulrich Christensen is at the Institut für Geophysik, Herzberger Landstrasse 180, 37075 Göttingen, Germany.

e-mail: urc@willi.uni-geophys.gwdg.de

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Immunology

Signal sequences stop killer cells

Eric O. Long

Most proteins destined for the surface of eukaryotic cells possess a signal sequence, which includes a stretch of several hydrophobic amino acids that guide protein translocation into the endoplasmic reticulum during protein syn-

thesis. The fate of amino-terminal signal sequences, found in certain transmembrane proteins, is thought to be much like that of ticket stubs: torn off by an endopeptidase and trashed by further proteolysis.

In an unexpected twist, Braud *et al.*, on

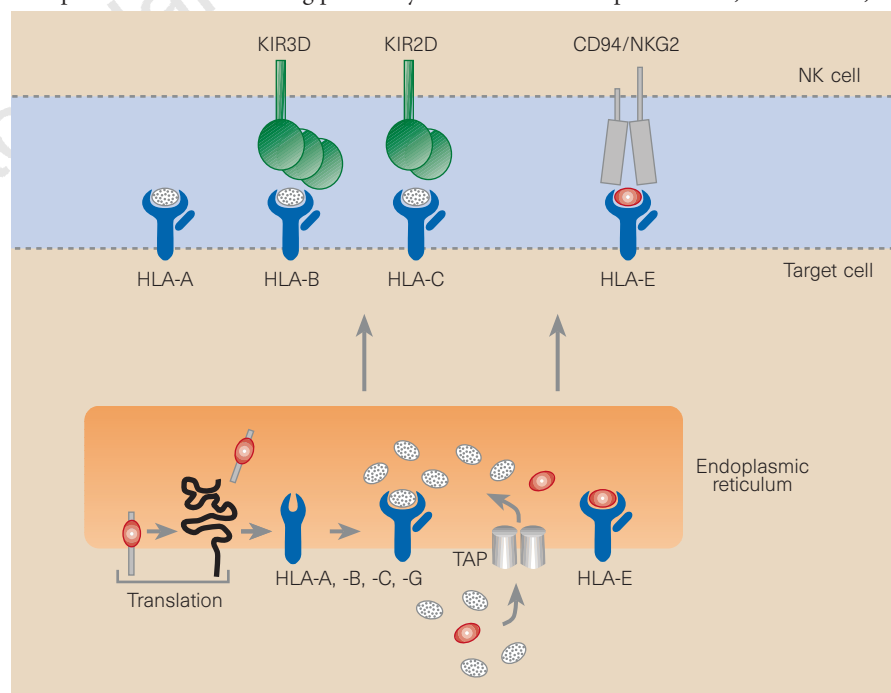


Figure 1 The ABC...E of natural killer (NK) cell tolerance to self. Two types of receptors specific for self HLA class I molecules inhibit NK cells, each having a distinct mode of HLA class I recognition. The lectin-like heterodimer CD94/NKG2 binds to HLA-E molecules¹, whose transport to the cell surface is limited by the availability and binding of a specific peptide (indicated in red) derived from signal sequences of other HLA class I molecules. The killer cell inhibitory receptors with three (KIR3D) or two (KIR2D) immunoglobulin domains recognize HLA-B and -C, respectively. HLA-B and -C bind many different peptides (stippled), most of which are compatible with recognition by KIR3D and KIR2D. The lower left portion of the diagram illustrates the first steps in synthesis of HLA class I molecules. An amino-terminal signal sequence directs translocation into the endoplasmic reticulum during translation of HLA class I messenger RNA. After cleavage of the signal sequence by an endopeptidase, the HLA class I polypeptide folds and assembles with β_2 -microglobulin and a short peptide delivered into the endoplasmic reticulum by the transporter for antigen presentation (TAP). Binding of the signal-sequence-derived peptide to HLA-E is also TAP-dependent⁴. The signal sequence of HLA-E lacks amino acids necessary for binding to HLA-E.

page 795 of this issue¹, and Borrego *et al.*, in a forthcoming issue of the *Journal of Experimental Medicine*², reveal a highly specific function in immune recognition for signal sequences of class I molecules of the major histocompatibility complex (MHC — a family of molecules well known to immunologists).

This finding opens a completely new perspective on the evolution and functional potential of signal sequences, which were previously thought to be rather inert.

Self–nonself discrimination is a central property of the immune system, and is of obvious importance in preventing destruction of the body's own tissues by killer lymphocytes such as cytotoxic T cells and natural killer (NK) cells. NK cells, which are easily activated upon binding to a target cell, are prevented from killing healthy cells of the host by inhibitory receptors. These inhibitory receptors are specific for MHC class I molecules, which are encoded by a family of genes that vary widely between individuals. Whereas MHC class I proteins turn off NK cells, they activate cytotoxic T cells, a more educated class of lymphocytes that achieve self tolerance by several different mechanisms. T cells recognize peptide fragments that are bound to self MHC class I proteins. Such peptides are derived from foreign proteins or from self proteins that failed to induce T-cell tolerance. Virus-infected cells and tumour cells that evade T-cell responses by interfering with MHC class I expression become targets for NK-mediated lysis.

The new findings^{1,2} provide a surprising link between NK cell tolerance to self and protein translocation mediated by signal sequences, and they explain the following, puzzling feature of NK inhibitory receptors. One type of inhibitory receptor used by human NK cells is a heterodimer of the polypeptides CD94 and NKG2 (ref. 3). Both are members of the calcium-dependent lectin superfamily. The CD94/NKG2 receptor seemed to react with products of different alleles of the HLA-A, -B and -C class I loci, as well as the invariant, nonclassical class I molecule HLA-G. This reactivity was perplexing because several related allelic forms of HLA-A and -B were not recognized.

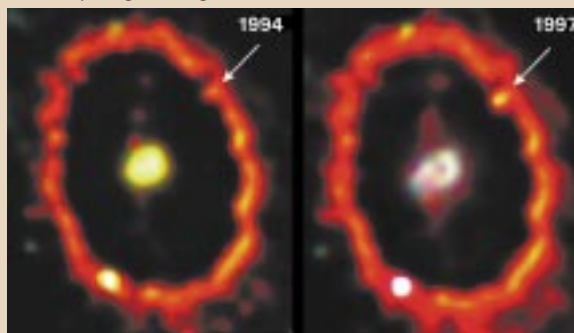
The CD94/NKG2 receptor now turns out to be specific for HLA-E, a nonclassical class I molecule. Expression of HLA-E at the cell surface depends on the binding of peptides derived from signal sequences⁴ of precisely those HLA class I molecules whose expression correlates with recognition by CD94/NKG2 (Fig. 1). The signal sequence of HLA-E itself, as well as those from several HLA-A and -B gene products, produces peptides that do not bind to HLA-E. Although specific for HLA-E, the CD94/NKG2 receptor is evidently designed to gauge the overall level of HLA class I expression on cells, which is one indicator of their health status.

Supernova 1987A

Shockwave hits the ring

About 170,000 years ago, a star exploded in the Large Magellanic Cloud, a satellite galaxy of the Milky Way. When light and neutrinos from this explosion reached the Earth in 1987, SN1987A became the brightest supernova seen since 1604 — and the best studied ever.

Eleven years later, a second light show is beginning. The blast wave from the original explosion has finally reached a dense and mysterious ring of gas surrounding the star. Its first contact can be seen in these Hubble Telescope images, which show a small patch of the ring suddenly brightening.



What produced this ring? It may have been thrown off by a merger between the main star and a binary companion, some 20,000 years before the explosion.

That idea is appealing because it could also explain why the progenitor star was a blue supergiant, instead of the red supergiant that usually precedes this type of supernova. But the ring could instead be the waist of a more extended shell of gas, emitted as a dense stellar wind in a passing stage of the precursor star's evolution.

Within a year or two we may know better. As the shockwave passes through at 18,000 km s⁻¹, it will heat the gas. The

ring will then shine brightly, allowing highly detailed spectroscopy and imaging, and illuminating gas elsewhere in the system.

It is a familiar story — often, the best way to learn about an object is to watch what happens when something hits it.

Stephen Battersby

The evidence for this mode of recognition through HLA-E is compelling. Braud *et al.*¹ show, first, that a soluble tetrameric form of HLA-E binds to CD94/NKG2 receptors on transfected cells. Second, they find that grafting an HLA-E-binding signal sequence onto an HLA-B molecule that lacks such a sequence permits surface expression of HLA-E and inhibition of NK cells expressing CD94/NKG2. Finally, Borrego and colleagues' results² indicate that recruitment of HLA-E at the surface of a transfected mouse cell by the addition of synthetic peptide ligands provides protection from lysis by NK cells expressing CD94/NKG2.

An analogy can be made in the mouse, where the nonclassical class I protein Qa-1 predominantly binds peptides from class-I-derived signal sequences⁵ that are very similar to those bound to HLA-E. The MHC class I proteins having suitable signal sequences predict that a receptor for Qa-1 analogous to CD94/NKG2 would have an apparent specificity for products of H-2D alleles. The approach described by Braud *et al.* may prove rewarding in the search for ligands of Qa-1 and other nonclassical class I proteins.

As well as CD94/NKG2, human NK cells also express a very different type of inhibitory receptor, named the killer cell immunoglobulin-like receptor (KIR). Single NK cells often co-express inhibitory CD94/NKG2 and KIR, suggesting that there is redundancy among inhibitory receptors³. But it now seems that the two receptor sys-

tems operate in distinct ways (Fig. 1). Different members of the KIR family mediate specific recognition of distinct groups of HLA-B and -C gene products. Therefore, NK cells that rely on KIR for inhibition can detect the loss of a specific MHC class I gene product, as occurs in some tumours. Many of the peptides among the vast array of those bound to HLA-B and -C proteins confer protection from lysis by NK cells that express KIR. The great variability in HLA class I genes between people suggests the need for a diverse repertoire of receptors that inhibit NK cells. Indeed, the predominant use of CD94/NKG2 for inhibition of NK cells in one person, but of KIR in another, was observed in two individuals having different sets of HLA class I ligands for these receptors⁶.

Several issues are raised by this unexpected involvement of signal sequences in NK cell tolerance to self. Every immune defence mechanism presents a challenge to pathogens. Certain viruses use specific strategies to interfere with MHC class I function or expression. An effective evasion tactic against both T and NK cells would be to selectively block peptide presentation by classical class I molecules, and to allow HLA-E to carry signal sequences to the surface. The selectivity of HLA-E for HLA class-I-derived signal sequences is not controlled by other properties of HLA class I molecules, because Braud *et al.* found that a class I signal sequence tagged onto an unrelated protein

was sufficient for recognition of HLA-E by NK cells. So, besides the presence of anchor residues for binding to HLA-E, unique features in HLA class I signal sequences must determine proper cleavage, transport and trimming for loading onto HLA-E. The capacity of HLA class I signal sequences to hold this amount of information sets a fascinating precedent for a similar regulation of cellular functions by signal sequences of other proteins. □

Eric O. Long is in the Laboratory of Immunogenetics, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852-1773, USA.
e-mail: elong@nih.gov

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Chemical dynamics

Noisy waves

Frank Moss

Random fluctuation, or noise, is familiar from communication, where a single parameter such as voltage varies with time. Usually it is a nuisance, but sometimes, because of the peculiar phenomenon known as stochastic resonance, it is a boon. Noise is also present in many extended natural phenomena: a number of interacting elements might be spread over a two-dimensional surface, each subject to local noise. Certainly we expect the noise to affect the dynamics of such a system — perhaps to a large degree — but are there systems in which the noise can increase some collective, or coherent, dynamical property? If a certain degree of noise were to maximally increase such a property, the process would be called spatiotemporal stochastic resonance (STSR). As Sándor Kádár, Jichang Wang and Ken Showalter report on page 770 of this issue¹, STSR has now been achieved experimentally for the first time — an achievement that may have implications for the workings of the brain.

The Belousov–Zhabotinsky reaction is a self-sustaining reaction–diffusion system, in which straight or spiral waves of activity can propagate in a thin film. Using a photosensitive version of the reaction, Showalter and colleagues applied spatiotemporal noise as part of a two-dimensional optical image. The image is of a rectangular region, divided into a large number of square subregions or cells, and the intensity of the light falling on a given

cell determines its state of excitability by controlling the rate of photoproduction of Br₂ ions (which are inhibitors of autocatalysis in this reaction). A high light intensity keeps the region below the threshold of excitability, with the result that disturbances are rapidly quenched. Conversely, with little or no light, disturbances grow into waves that propagate indefinitely².

The average light intensity for all cells was adjusted to maintain the entire region in the subexcitable state, so that indefinitely sustained waves were impossible. To this average, time-dependent noise was added. Waves propagating into the region from an external source lived longer, and so propagated further, in the presence of this spatially distributed, time varying noise. An optimal noise intensity resulted in sustained waves. Adding still more noise worsened wave propagation and broke up the waves into segments of random lengths. Spiral waves could also be induced (Fig. 1a).

The authors define a signal strength for the propagating waves: the ratio of the length of coherent wave segments as they pass a certain point in space relative to their lengths on entering the subexcitable region. The signal strength passed through a maximum as the noise intensity passed through the optimum value — the signature of stochastic resonance^{3,4}.

These results are successfully simulated using a modified ‘Oregonator’ model⁵, so

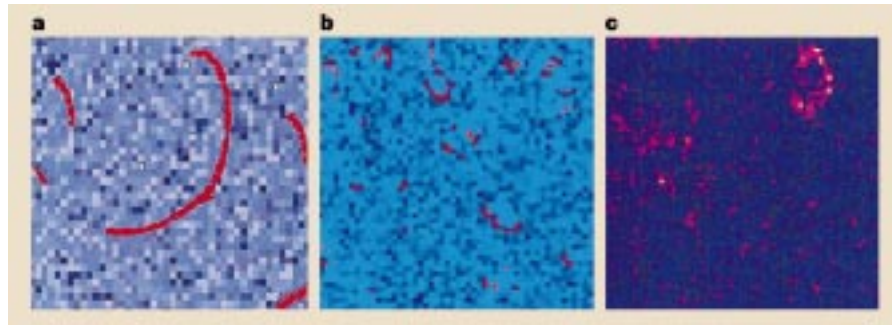


Figure 1 Noise-mediated spiral waves. Waves excited by noise; a, in the Belousov–Zhabotinsky chemical reaction; b, the ‘Oregonator’ numerical model; and c, a cultured network of brain tissue (astrocyte syncytia).



100 YEARS AGO

We have received further correspondence relating to the two Societies in Lincolnshire, to which reference was made in our issues of December 30 and February 3. It appears that the older Society, the Lincolnshire Naturalists’ Union, does not regard with unmixed friendliness the newer and possibly more vigorous Science Society. Into this unfortunate conflict of interests it is not our province to enter, and we can only repeat with renewed emphasis that it is a serious mistake to allow the spirit of rivalry to enter into the matter at all. The welfare of both Societies can only suffer, and the progress of science in the county can only be retarded by friction. The Lincolnshire Science Society explains its origin by accusing the Union of failing to carry out the objects for which it was founded. There may or may not be truth in the accusation, but we are bound to admit that evidence of scientific activity on the part of the Union has not been obtainable. We cannot find the latter body among the corresponding societies of the British Association; neither can we learn that any publication has been issued under its auspices.... We can only hope that Lincolnshire will not present to the scientific world a divided front on a question in which both parties are really striving for the same end.

From *Nature* 17 February 1898.

50 YEARS AGO

On November 12, 1947, it came my way to spend, alone, a couple of hours in the evening with the late Prof. [Alfred North] Whitehead, and Mrs. Whitehead, in their apartment within a stone’s throw of the centre of Harvard. It seems likely, therefore, that I was the last person from Britain to see the great man before he died. We talked of Trinity (“Such a good place”, he whispered) and its ‘characters’, of whom he spoke affectionately, coupled with an occasional sly dig, as entrancing as it was kindly. He was devoted to the country of his adoption: a remark stands out in my memory — “The Americans have a streak of tenderness, yes, how valuable that is these days”. Many people have used gracious words about our trans-Atlantic cousins, but it was left to him to state their noblest trait, and the one holding out hope for the world.

From *Nature* 21 February 1948.

named after the place of its invention in Eugene, Oregon. The model is a set of three partial differential equations that describe the reaction–diffusion process. Showalter and colleagues added a term to account for the photosensitive generation of bromide ions, and predict wave propagation patterns remarkably similar to those observed in the experiment. Before this experiment, STSR had been studied only theoretically or by numerical or electronic simulation in one-dimensional sets of coupled^{6,7} and uncoupled⁸ elements, and in two-dimensional arrays of threshold elements⁹. But those 2D simulations, in spite of their simplicity, mimic all the features of the present experiment.

The implications of the present experiment extend far beyond chemical dynamics. Spiral waves, spontaneously generated by noise, have also been simulated with the Oregonator (Fig. 1b). They are strikingly similar to recent observations of noise-initiated and sustained long-range coherent waves of calcium ions in cultured brain tissue¹⁰ (Fig. 1c) indicating a similar under-

lying dynamical process. The possibility that calcium waves transmit or coordinate information over centimetre distances in glial cell networks (that is, in the brain) has already been suggested, but the role of noise remained obscure. Now that noise-sustained spiral waves have been observed in a well characterized chemical system, we can speculate that spatiotemporal noise may be an important feature of the brain's working. □

Frank Moss is at the Center for Neurodynamics, University of Missouri at St Louis, St Louis, Missouri 63121, USA.

e-mail: mossf@umslvma.umsl.edu

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Functional genomics

Double-stranded RNA poses puzzle

Richard W. Wagner and Lin Sun

The human genome is predicted to contain between 50,000 and 100,000 genes¹. To work out what these genes do, an array of techniques is needed to evaluate the protein–protein interactions and biochemical pathways of any gene product. The nematode worm *Caenorhabditis elegans* is an excellent system for such studies because of its well-understood genetics and development, evolutionary conservation to human genes, small genome size and relatively short life cycle. The 100-megabase-pair genome will be completely sequenced this year, and a total of 17,000 genes have been predicted, many with human counterparts. Approaches used to manipulate gene expression in *C. elegans* include transposon-mediated deletion², antisense inhibition³ and direct isolation of deletions after mutagenesis^{4,5}. Although these methods have proved useful, limitations still exist.

On page 806 of this issue, Fire and colleagues⁶ describe a remarkable and surprising technique for inhibiting gene function in *C. elegans*. They turned off a specific gene in progeny worms by microinjecting double-stranded RNA (dsRNA) complementary to the coding region of the gene into the gonads of adult animals. Using a well-characterized gene, *unc-22*, which encodes a non-essential myofilament protein, they showed that injection of dsRNA produced a phenotype

characteristic of *unc-22* inhibition — twitching.

In a series of well-controlled studies, the authors also found that injection of dsRNA targeted to a reporter gene for green fluorescent protein resulted in a dramatic — and

specific — decrease in protein production. Furthermore, when they injected dsRNA targeted to another gene, *mex-3*, the result was a loss of *mex-3* RNA in early-stage embryos. In other words, at the levels of phenotype, RNA and protein, the interference with gene expression was specific and reproducible.

Perhaps most astounding is the phenomenon that the dsRNA causes gene inhibition. Previously³, Fire and co-workers had been puzzled by the fact that antisense RNA alone — which is often used to inactivate sense messenger RNA — was only marginally effective. Furthermore, results using the antisense RNA were mimicked by injection of sense RNA, a control in their studies. They later found out that these data could be largely explained by an artefact of the transcription process that was used to generate the antisense and sense RNAs; namely, dsRNA fragments.

Additional experiments by Fire *et al.*, designed to shed light on the possible mechanism of the dsRNA-mediated inhibition, painted an even more mystifying picture. For example, even when only a few copies of the dsRNAs are present in each cell, they are active against highly abundant RNAs. This indicates that the interference occurs either by a catalytic mechanism or at the chromosomal level — and not by a conventional antisense mechanism. The authors also found that only dsRNAs that are complementary to coding regions of the gene are active, and not, for example, those targeted to introns or promoter regions. This argues against a generalized mechanism involving chromosomal inactivation, such as chromosomal deletion. Moreover, dsRNA interference seems to cross cellular boundaries with

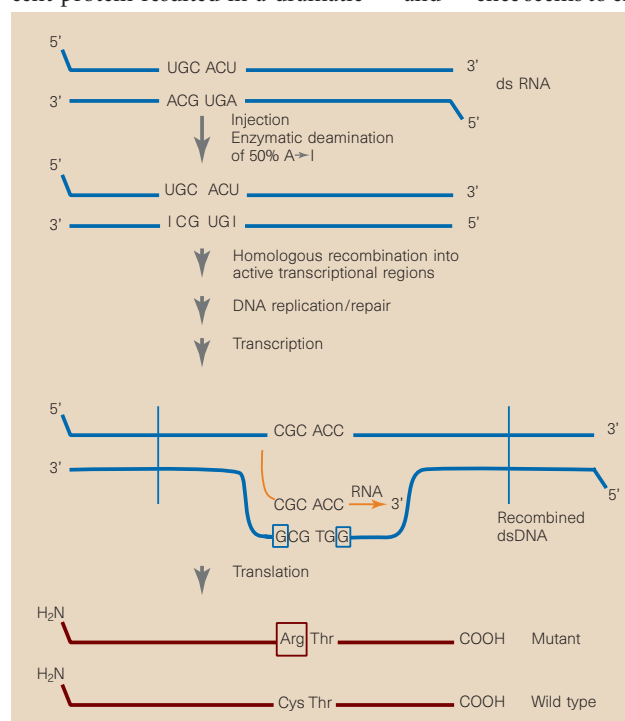


Figure 1 Possible mechanism for inhibition of gene expression in *C. elegans* by double-stranded RNA. Fire *et al.*⁶ have convincingly shown that, at the phenotype, RNA and protein levels, dsRNA-mediated interference with gene expression is specific and reproducible. Perhaps, on injection into worms, dsRNA is modified by dsRNA adenosine deaminase. Transfer of this information back into the chromosome may occur by a recombination event. After replication and mismatch repair, transcription and translation result in mutant proteins that have impaired function.

ease. Gene inhibition was observed in progeny when dsRNA was injected into the body cavity of the adult (gonadal injections had been thought to be necessary), and in somatic tissues of young adults after injection into their body cavity.

What kind of mechanism have Fire and colleagues uncovered? This is not the first puzzle posed by dsRNA. Almost ten years ago, Bass and Weintraub⁷ and Wagner *et al.*⁸ discovered an enzyme that binds dsRNA and deaminates adenosines in the duplex to inosines. After a feverish hunt for the cellular function of the dsRNA adenosine deaminase, it was found to be involved in the post-transcriptional editing of messages. Inosines are read by the cellular machinery as guanosine, so the enzyme could alter the genetic make-up of mRNA (reviewed in refs 9, 10).

Could this dsRNA adenosine deaminase be involved in a complicated pathway that results in gene inhibition in *C. elegans*? Quite possibly. The enzymatic activity has been found in *C. elegans*, and would probably treat the injected dsRNA as a substrate. A specialized homologous recombination system would be needed, which would use the modified dsRNA to transfer the genetic alterations into the chromosome (Fig. 1).

This model fits some of the data: modification of adenosines to inosines alters the genetic make-up of the injected dsRNA; transfer of this information into the genome by recombination would affect coding (but not intronic) regions; and mutations introduced by the inosine substitutions would affect the ability to detect mRNA and, at least partially, the function of the protein. These mutations could account for the surprising result that only a few copies of dsRNA are required per cell, because they would have an effect at the level of the chromosome. Of course, such a model is a stretch of the imagination and is not supported by all of the data. For example, attempts to use homologous recombination with dsDNA in *C. elegans* have largely failed³.

Fire and colleagues⁶ have uncovered a complex and intriguing mode of regulation in *C. elegans*. Does dsRNA perform a biological function in *C. elegans* (and is this function titrated out by the microinjected dsRNA)? Does a similar phenomenon exist in other organisms? What would happen if transgenic animals or plants were generated expressing both the sense and antisense strands of a transgene? A similar mode of action would not be suspected to occur in mammals, because injection of dsRNA is often used as a control for antisense experiments, albeit at the individual cell (and not organism) level. Nevertheless, perhaps specific 'knockouts' can be generated this way, for organisms in which genetic material cannot be delivered by microinjection. Whatever the mechanism might be, dsRNA-

mediated inhibition of gene expression will provide a useful alternative for working out gene function in *C. elegans* and, maybe, in other animals and plants. □

Richard W. Wagner and Lin Sun are at Phyllos Inc., 300 Putnam Avenue, Cambridge, Massachusetts 02139, USA.

e-mail: rwagner@phyllos.com

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Liquid crystals

New designs in cholesteric colour

Peter Palffy-Muhoray

Since their discovery in 1888, cholesteric liquid crystals have been subject to considerable attention, resulting in applications in ink and paint technologies, flat-panel displays and thermal imaging. Writing in *Advanced Materials*¹, Tamaoki and co-workers describe a new technique for rewritable full-colour image recording on thin cholesteric films. The low-molecular-weight compound they have developed for this purpose is a cholesteric glass, which is stable at room temperature and which could have applications in optics as well as infor-

mation display and storage.

The optical properties of cholesterics have made them useful in display^{2,3} and laser technologies⁴ as well as in the visual arts⁵. In reflected light, cholesterics show intense iridescent colours with a metallic sheen, as seen on scarab beetles. In these materials, rod-like molecules are orientated, on the average, parallel to one another in a given plane, so that the direction of orientation varies linearly with position in the direction normal to the plane. This results in a spatially periodic twisted helical structure as shown in

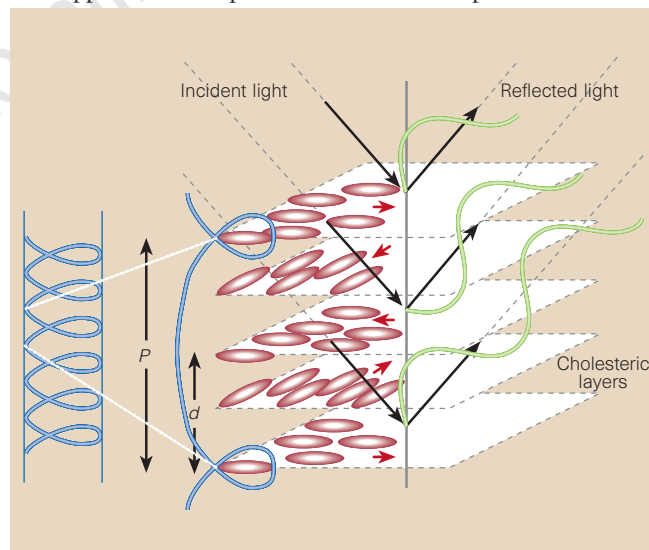


Figure 1 Sketch of cholesteric structure, showing the dependence of molecular orientation on position. The tip of a vector indicating local molecular orientation traces out a helix. Reflected light waves satisfying the Bragg condition emerge in-phase and add constructively. In the work discussed here, Tamaoki *et al.*¹ have developed a cholesteric glass that is rewritable and stable at room temperature (see Fig. 3).

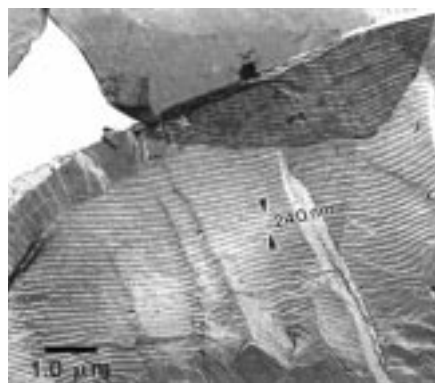


Figure 2 Transmission electron micrograph of freeze-fractured helical cholesteric. The pitch is 240 nm. (From ref. 12.)

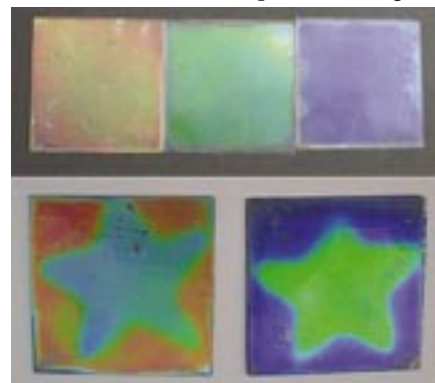


Figure 3 Photographs of thermally addressed and quenched cholesteric solid films. (From ref. 1.)

Fig. 1, which can give rise to strong Bragg reflection. This occurs in a band of wavelengths $\Delta\lambda = P\Delta n$, where $P=2d$ is the pitch, d the spatial period, and Δn the birefringence. The first-order wavelength $\lambda = P(n_{\text{eff}}^2 - \sin^2 q)^{1/2}$, where q is the angle of incidence, and n_{eff} the effective refractive index. The electron micrograph of Fig. 2 shows the periodic structure of a cholesteric liquid crystal quenched to the glassy state.

Although the microscopic origins of pitch are still a subject of study⁵, it is well known that the pitch and the reflected colours depend on temperature. Liquid-crystal temperature indicators are based on this effect. For their full-colour recording on cholesteric films, Tamaoki *et al.* have synthesized a low-molecular-weight cholesterol derivative, which, on cooling, shows a cholesteric liquid-crystal phase in the temperature range 87–115 °C between the isotropic liquid and the crystalline solid. Their recording technique consists of first forming a coloured image on their cholesteric liquid-crystal film by cooling it to different temperatures at different points, then rapidly cooling to a temperature below its glass-transition temperature of about 80 °C. The rapid cooling prevents crystallization, and preserves the cholesteric structure and the colour. The system can be repeatedly rewritten. Coloured 'pixels' and sample images recorded by this method are shown in Fig. 3.

Two technologically relevant questions about cholesterics are how to adjust the pitch, and how to keep it constant once it has the right value. As well as being sensitive to temperature, the pitch depends on the concentration of chiral constituents. High-resolution spatial patterning of pitch and colour can be achieved by ultraviolet irradiation of samples with photosensitive chiral dopants⁶. But although this technique has great potential for display applications, it is not suitable for rewritable recording because it is not reversible. Alternative ways of locking the pitch are by polymerization^{7,8}, or by using a polymer network to stabilize the cholesteric structure⁹. In its 'Designo' programme, Mercedes-Benz uses cholesteric liquid-crystal polymers as special-effects paints for its cars. Polymer-stabilized low-molecular-weight cholesterics, with an internal network of photopolymers, are used in bistable reflective display applications³. But again, these schemes are not suitable for rewritable recording by altering the pitch because of their irreversibility.

The optical properties of cholesterics change slightly on quenching from the liquid crystal to the glass; the reflection band is typically broadened and shifted because of a decrease in pitch due to thermal contraction, and because of the change in the refractive indices (T. Kosa, personal communication). Unique features of Tamaoki and colleagues' material are its extremely broad reflection

band, which probably originates in a strongly anisotropic molecular polarizability and allows the realization of colours over virtually the entire visible spectrum; and its high glass-transition temperature, which allows the glass to remain stable at room temperature.

In their paper, Tamaoki *et al.* discuss the possibility of laser-addressing their material by using laser heating due to absorption. Laser writing, where the local structure of the material is altered by light from a laser, has been carried out in a variety of liquid crystals, including polymer-stabilized cholesterics^{10,11}, but by exploiting different mechanisms. Tamaoki and co-workers' system offers the possibility of laser-writing high-resolution full-colour images on solid cholesteric glass films, for image recording and for high-density storage of optical information.

Another promising application of room-temperature cholesteric glasses is in optical elements for high-power laser systems. Elements in use today confine the liquid crystal between glass plates⁴, which may bow and

distort the wavefront. Because the new cholesteric glass is solid, optical elements using Tamaoki and colleagues' material could be set on a single substrate to overcome this problem. □

Peter Palfy-Muhoray is at the Liquid Crystal Institute, Kent State University, Kent, Ohio 44242, USA.

e-mail: mpalfy@cpi.kent.edu

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Ice sheets

On the shelf

Over three-quarters of Earth's fresh water is locked in the great ice sheets covering Greenland and Antarctica. The stability of these ice sheets is the single largest unknown factor in attempts to predict how sea level may rise in the future, as even a small change in the mass balance of this ice would result in a significant change in global sea level. The recent break-up of some ice shelves on the Antarctic Peninsula (see *Nature* 379, 328–331; 1996), and the recognition that large changes have occurred over the past few decades in ice streams feeding the



West Antarctic ice sheet (*Science* 279, 689–692; 1998), has generated a great deal of public interest in the problem.

Although the break-up of ice shelves does not directly affect sea level (by definition, ice shelves are the floating parts of ice sheets, and so already displace an equivalent volume of sea water), it could be that their disintegration would seriously affect the discharge rate of grounded ice from inland regions.

Elsewhere in this issue (*Nature* 391, 778–780; 1998), Christopher Doake and colleagues describe an attempt to establish a stability criterion for ice shelves through modelling of the two northern sections of the Larsen ice shelf on the Antarctic Peninsula. Shown here is the northern section of this ice shelf, Larsen A, which disintegrated within a few days in January 1995 (just before this photograph was taken); in the upper left-hand corner can be seen the edge of Larsen B, which is still intact, although retreating. Through finite-element computer modelling of the strain rates for these two sections of ice shelf from 1986 to 1997, Doake *et al.* discovered that only the initial and final ice front positions of Larsen A were stable, and that if Larsen B were to retreat only a few more kilometres it too is likely to disintegrate.

The hope is that these stability criteria can be used elsewhere, to help distinguish which ice shelves are in danger of collapse.

John VanDecar

Neural patterning

Deconstructing the organizer

A. Ruiz i Altaba

Modern understanding of animal development depends on the concept of the 'organizer'—a group of cells which sends signals that induce distinct fates in neighbouring cells, resulting in spatial patterning¹ (see box). For example, initial patterning of the central nervous system, along the anteroposterior (forebrain-to-spinal-cord) axis seems to depend on signals from the organizer². Recent experiments, however, reveal a complex series of signalling events, indicating that anteroposterior patterning of the neuraxis does not depend on a single organizer. Rather, patterning may be due to the concerted action of a series of organizing centres. Further evidence to support this is given by Houart *et al.*³ on page 788 of this issue. They have identified an organizing centre involved in brain patterning which—in contrast to the classical organizer, which occupies a posterior position relative to the neural ectoderm—is located at the prospective anterior end of the zebrafish neuraxis.

According to the old model for anteroposterior patterning, all neural tissue is induced by the underlying, anterior axial mesoderm (the anterior midline cells of the middle layer of embryonic tissue). Thus, all neural tissue first has anterior (forebrain) character¹.

By contrast, in the planar model^{2,4}, neural

anteroposterior pattern is induced by signals from the organizer, acting through the plane of the neural ectoderm^{2,5,6} (part of the outer embryonic layer; Fig. 1a). In both models, other, 'posteriorizing' signals (possibly from adjacent non-axial tissue⁷; Fig. 1b) may contribute towards specifying posterior neural fates.

Two aspects of the planar model remain controversial. First is the possibility that the anterior ectoderm affects the character of newly induced neural tissue in frog ectodermal explants⁴, to account for the anterior character of this tissue. Second is the proposal that anteroposterior patterning of the neural plate is akin to that of a developmental field, with planar signals emanating from its boundaries—from the organizer posteriorly, and from a putative boundary zone anteriorly⁴.

Houart *et al.*³ now show that an early anterior neural boundary organizer (ANB) exists, and that it is required for normal development of the brain (Fig. 1c). The authors transplanted small groups of cells from the anterior border between neural and non-neural ectodermal tissue, to more posterior positions within the neural plate of midgastrula-stage zebrafish embryos. They found that anterior row-1 neural cells (that is, the line of cells just posterior to the border) can re-specify the fate of surrounding

host cells, to express anterior forebrain markers. Row-1 cells (the ANB) are also required for normal expression of early markers for the anterior forebrain and, unexpectedly, the later survival of most of the brain.

The activity of the zebrafish ANB cells is similar, in part, to that described for the anterior neural fold of early somite mouse embryos⁸. However, the ANB acts during gastrulation at pre-somitic stages. Moreover, fibroblast growth factor 8 (which is expressed in the mouse anterior neural fold, and can mimic its effects⁸) is not expressed in fish embryos by the time the ANB acts³. Thus, the results of Houart *et al.* functionally define a hitherto unknown organizing centre at the anterior neural edge in the zebrafish embryo. The finding that the ANB must act through planar signals on more posterior neural cells further supports the planar model.

Is the zebrafish ANB present in other species? Anterior visceral endodermal (AVE) cells of the early mouse gastrula may have a similar role⁹ (Fig. 1d). However, these two organizers are unlikely to be equivalent, because homologues of genes that are expressed in the mouse AVE are not found in the zebrafish ANB. But, in the mouse, the anterior boundary of neural and non-neural ectodermal cells overlies the AVE, so perhaps these ectodermal cells are the mouse counterpart of the zebrafish ANB. In frogs, this boundary could be found near the animal pole, and in chicks it would be found in the anterior epiblast, distal from Hensen's node.

Conversely, is there an AVE-like organizer in fish and frogs? Some genes that are expressed in the organizer of fish and frogs are also expressed in the mouse AVE. So it is possible that mice have split the early frog and fish organizer into AVE and the node—with AVE involved in anterior patterning, and the node in posterior patterning. This dichotomy is consistent with the existence of early and late organizers in amphibians¹. The early gastrula (or head organizer) can induce a complete axis after transplantation, whereas the late gastrula (or trunk organizer) can induce only trunk structures.

In frogs, the *Cerberus* gene¹⁰ encodes a

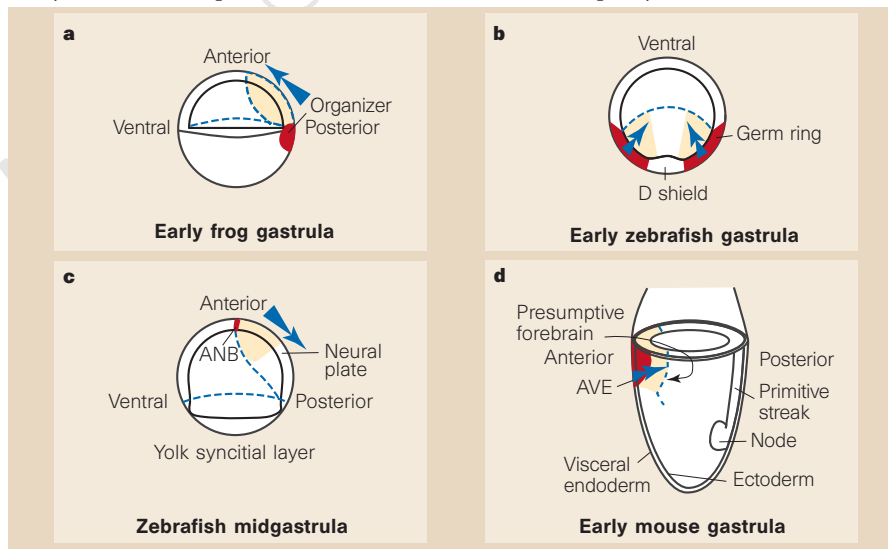


Figure 1 Induction of neural anteroposterior patterning by distinct organizing centres in early vertebrate embryos. Red depicts organizing centres, and yellow depicts affected regions. Blue arrows depict patterning/inductive signals. a, Side view of an early gastrula frog embryo, showing the position of the organizer in the dorsal side and the action of planar signals that affect the forming neural ectoderm^{2,5,6,12}. b, Top view of an early gastrula zebrafish embryo, showing the action of posteriorizing planar signals from adjacent germ-ring non-axial tissue. c, Side view of a midgastrula zebrafish embryo, showing the anterior neural boundary organizer (ANB) in the anterior neural plate. d, Side view of an early streak mouse gastrula, showing the anterior visceral endodermal cells (AVE), adjacent to the prospective forebrain.

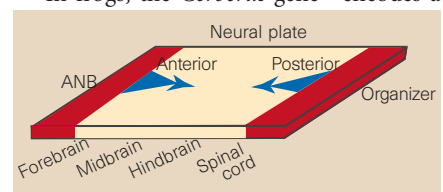
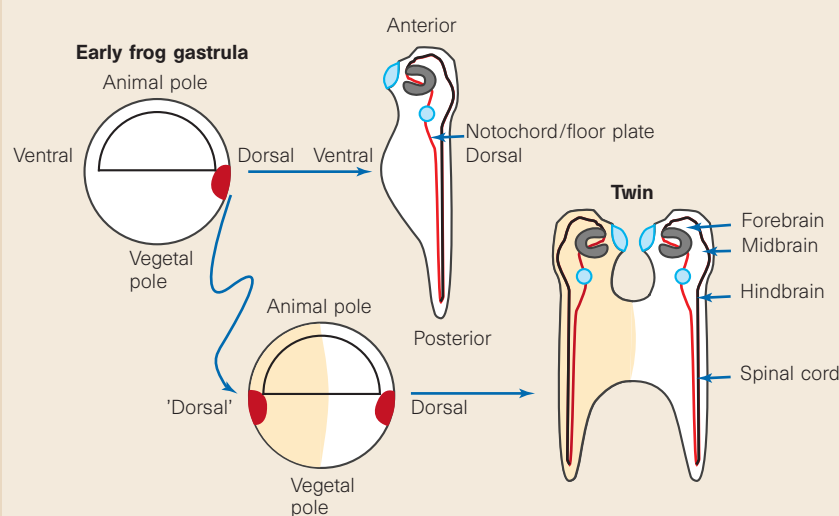


Figure 2 Planar model for initial anteroposterior patterning in the vertebrate neural plate. Early anteroposterior patterning seems to occur by the action of planar signals from the anterior³ (the ANB) and the posterior (the organizer) boundary regions, with the neural plate behaving as a developmental field⁴.

Origins of the organizer



The organizer was defined over 70 years ago by Hilda Mangold and Hans Spemann, in grafting experiments with amphibian embryos¹. When a small group of mesodermal cells of the dorsal lip (which first begin gastrulation movements) is grafted to the ventral side of a host sibling embryo, this develops a complete twinned body, with two perfectly organized head-to-tail axes. Because the graft gives rise to only a small part of the secondary axis, dorsal lip cells (and descendants; red)

were proposed to be endowed with 'organizing' properties that induce a change in the fate of surrounding cells (yellow). This, and similar results obtained in a variety of species¹, together with the evolutionary conservation of the axial structures, indicates that the organizer – the dorsal lip in amphibians, the shield in fish and Hensen's node in birds and mammals – is a central source of patterning information for the formation of the vertebrate body plan.

secreted head-inducing factor that is expressed in the anterior endodermal component of the early organizer, but not in the late organizer. And in mice a *Cerberus*-like gene is expressed in the AVE but not in the node¹¹.

It is possible that, in mice, an ANB is induced by the AVE. Likewise, the ANB in fish and frogs may be induced by the 'AVE-like' cells of the head organizer (or even Nieuwkoop's centre or the yolk syncytial layer).

Alternatively, a putative ANB in mice could be a distinct entity that overlies the AVE, the two organizers having temporally distinguishable signalling properties. Induction of a putative ANB by the organizer in frogs could account for the induction of forebrain markers by planar signals¹². The function of an early ANB organizer in frog embryos could also explain why all newly induced neural tissue in animal cap ectoderm has an anterior (forebrain) character. Moreover, an endogenous ANB could synergize with a transplanted organizer, to create the complete anteroposterior pattern in the twinned axis (see box).

How these early organizing centres are induced, and what molecules mediate their actions, are questions that need to be answered to understand the logic and mech-

anisms of neural anteroposterior patterning.

The emerging view is that interrelated and interdependent organizing centres located at boundary regions, in particular the anterior (the ANB) and posterior (the organizer) boundaries, pattern the neural plate via planar signals (Fig. 2). In this sense, the early midbrain/hindbrain boundary¹³ represents yet another organizing centre involved in later neural anteroposterior patterning. □

A. Ruiz i Altaba is at the Skirball Institute, Developmental Genetics Program, NYU Medical Center, 540 First Avenue, New York, New York 10016, USA.

e-mail: ria@saturn.med.nyu.edu

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Daedalus

Winter in slumberland

Winter is a depressing time, particularly for old people trying to keep fed and warm on an inadequate pension. The British government makes special payments to such folk in cold weather. In the face of similar winter hardships, many animals simply hibernate. They allow their body temperature to fall to some low value, and their metabolism slows to a crawl. Even big mammals, such as bears, hibernate. So Daedalus is now exploring the possibilities of human hibernation.

Cold has two competing effects on the human body. The metabolic rate slows down, reducing the demand for oxygen and fuel; but at the same time, the heart and lungs slow down as well, reducing the supply of these essentials. For a few degrees of cooling, these effects stay in balance. Surgical patients can be cooled slightly to extend the time for which the heart or brain can be isolated. But at about 25 °C the balance breaks down; the lungs may keep going but the heart is likely to stop. To become practical, human hibernation will have to keep the blood moving somehow.

The obvious idea, fitting the heart with a pacemaker and forcing it to beat, seems not to work — at least, not in rabbits. But Daedalus recalls that volunteers in centrifuges, or those subjected to negative pressure below the waist, can have as much as 20% of their blood drained down and 'pooled' in the lower part of the body. So DREADCO engineers are devising a sort of 'iron heart', by analogy with the iron lung. Each of the subject's limbs is placed in a sealed cylinder, and big sealed cups are placed strategically against his body. By sucking and blowing on these containers in a suitable rhythm, the blood can be shunted peristaltically from region to region around his body. The iron heart will never approach the pumping efficiency of the real thing. But it should circulate the blood sufficiently to meet the greatly reduced oxygen demand of a hibernating pensioner.

A refrigeration unit and various monitoring controls complete the DREADCO Hibernator. The user will be placed in the device and chilled into dreamless oblivion. Like a hibernating animal, he may need to be woken at rare intervals to eat and excrete; but essentially winter will cease to exist for him. And, of course, he will cease to exist for the government. In the municipal Hibernatorium, chilled pensioners in bulk will sleep the winter away at trivial cost to the Nanny State.

David Jones

Obituary

Kenichi Fukui (1918–98)

Theoretical chemist who had fundamental insights into chemical reactivity

Kenichi Fukui, the first Japanese Nobel laureate in chemistry, died on 9 January this year. When Fukui was born, the electronic theory of chemical bonding was in its infancy. Oh, chemists did have a very well-grounded chemical notion of a bond and a symbolism for it. But the electron itself had barely reached maturity, and Lewis and Langmuir were just assimilating the then new Bohr quantum theory of the atom into chemistry.

By the time Fukui completed his education in 1941, things were different. The first textbook of quantum chemistry had been written six years before by a German in exile, Hans Hellmann. And American and British chemists had secured a place for quantum mechanics in chemistry, through the charismatic expositions of Linus Pauling, the quieter and deep reflections of Robert Mulliken, and the elegant, perceptive teaching of Charles Coulson.

Fukui wrote that chemistry was not his favourite subject in high school. His father (the family is an old Nara one), however, enquired of Gen-itsu Kita, a professor at Kyoto Imperial University, what his bright son might study. Kita suggested that Kenichi should enter the department of industrial chemistry. In those days children listened to the advice of their elders, and Fukui graduated from that department in 1941.

Some important work on synthetic aviation fuels during the Second World War led to an appointment in the department of fuel chemistry, in the faculty of engineering of Kyoto Imperial University. Much productive experimental work on reaction engineering and catalysis followed. The building of a career in an applied setting was, I believe, crucial — it sensitized Fukui to problems of real chemical reactivity. In this he had an advantage over his 'purer' theoretician colleagues. It is also an interesting counterpoint to the generally assumed inflexibility of Japanese universities and the *koza* system that Fukui could, in the end, do what elsewhere would be labelled as 'pure' chemistry in an applied setting.

For this young scientist had all along had theoretical aspirations and talents. He told me that he read Dirac's quantum theory text in the dull moments (I never found out if there were many or few)



between exploding American bombs. After the war, he gathered around himself a talented group of theoretical students, and in 1952 published in the *Journal of Chemical Physics* the paper that defined the idea of frontier orbitals.

Erich Hückel's seminal ideas on the stability of π -electron systems were finally receiving their due in just those years, and simple molecular orbital theory was in the ascendancy. Chemistry *is* reactivity; so it was natural to think of reducing the surfeit of information in the wavefunctions (how much worse it is now!) to a few pithy numbers, indicators of reactivity.

Various reactivity indices were devised, most emphasizing the charge distribution in the molecule as a whole. Fukui, Teijiro Yonezawa, Chikayoshi Nagata and Haruo Shingu's brilliant simplifying idea was to concentrate on one orbital — the highest occupied molecular orbital of the molecule when the reaction was with an acid; the lowest unoccupied molecular orbital when the reaction was with a base; and the singly occupied molecular orbital for a radical reaction. They called these orbitals the frontier orbitals of the molecule.

This notion, of tracing the essence of basicity or acidity or radical reactivity to a single orbital, was so simple! And, remarkably, it worked. The underpinnings of the idea were easily found by Fukui in perturbation theory, the natural language of quantum mechanics. In time, he and his co-workers expanded their ideas into a wide-ranging theory of orbital interactions and reactivity.

I first met Kenichi Fukui in early 1964.

In the very next year our lives were inextricably bound together, when R. B. Woodward and I developed the concept of orbital symmetry control of organic reactions. Ours was a primitive frontier orbital approach, developed intuitively and graphically, and buttressed by simple molecular orbital calculations. Our idea of a controlling orbital was in the spirit of Fukui. And indeed Fukui could right away derive all of our conclusions. What happened next was interesting, in that the success of the orbital symmetry ideas very naturally heightened, retrospectively, the respect of the world community for Fukui's frontier orbitals.

For Kenichi Fukui and me, science for once worked the way it should on the human level — instead of a competition there developed a friendship. Part of our bond was formed by the four talented Fukui graduate students who then were postdocs with me. Kenichi and I were brought together not by the accident of shared external recognition, but by a real kinship in spirit — yes, across cultures, and respectful of the cultural differences that enrich life — and by a shared philosophical outlook and scientific style.

In time he and I were awarded the Nobel prize in chemistry. The event had more serious consequences for Kenichi than for me. In the United States the eye of the public is on rock stars, and Nobel prize winners are relatively many. So — thank God — we are left alone. In Japan, Fukui, as the country's first chemistry laureate, had a tremendous burden of committees, official obligations and public appearances. It was not easy for this shy intellectual gentleman of the old school to face his duty. But he did so, with style.

Kenichi Fukui was impelled by the truest desire to understand this world, and especially our dear chemistry. He strove to do so in pictures of conceptual simplicity and real depth. And he understood so well that the nature we have tried to comprehend (and what our creation has added to it) is a precious gift, unique, and worth preserving.

So I miss him, as does our chemical community. I remember our first encounter on Sanibel Island, precisely 34 years ago. And, among many subsequent meetings, one shared moment rises vividly — together we watched a great Kyoto ceramic master, Miyashita Zenju, his old hands trembling, then steadying as he put them on the clay turning on his wheel.

Roald Hoffmann

Roald Hoffmann is in the Department of Chemistry, Cornell University, Ithaca, New York 14853-1301, USA.
e-mail: rh34@cornell.edu

Boccioni's ballistics

Viewpoints revolve in space and are transformed in time in the art of Umberto Boccioni, a member of the Futurists, who embraced the new sciences and technologies of their age. Boccioni aimed to express a series of relativities.

Martin Kemp

A new art for a new age. Such was the refrain that ran through avant-garde art, from the time in the nineteenth century when Baudelaire (1821–67) announced his search for a “painter of modern life”. By 1900, the “modern life” was being defined by the remorseless spread of the new technologies of energy, transport and communication, and by new notions of time and space

Such new ideas included the non-Euclidean geometry developed by Bernhard Riemann in 1854, and relativity, introduced by Einstein in 1905. The 1907–1912 pictorial revolution of the Cubists Pablo Picasso and Georges Braque involved the dissolution of perspectival space into fractured, interpenetrating planes and plural viewpoints. Retroactively — and none too convincingly — their achievement was hailed as the realization of Riemann’s non-Euclidean geometry and even as the embodiment of Einsteinian relativity.

The Futurists in Italy pushed the Cubists’ pictorial reforms in the direction of the new sciences and technologies. In “Foundation and Manifesto of Futurism” in 1908, the moustachioed poet F. T. Marinetti belligerently advocated the destruction of museums, the “cemeteries” of art, and lauded the beauty of “a racing car whose hood is adorned with great pipes, like serpents of explosive breath” over the most revered sculptures of antiquity.

Umberto Boccioni, the most creative and versatile of the Futurist artists, was the leading voice behind the “Technical Manifesto” produced by the Futurist painters in 1910 and sole author of their sculptural manifesto two years later. His passionate writings were liberally laced with neologisms, printed in capitals:

**UNIVERSAL DYNAMISM,
INTERPENETRATION OF PLANES,
EXTERIOR and
INTERNAL PLASTIC INFINITE,
PHYSICAL TRANSCENDENTALISM,
INVISIBLE INVOLVING SPACE.**

These alluded, poetically as much as technically, to current mathematics and physics.

His work involved little direct mastery of the technicalities of the new sciences, but rather an intuitive translation of what was sensed to be the crux of developments, not least as translated by the philosopher Henri Bergson. Indeed, Bergson’s *Creative Evolution* (1907) argued that “intelligence”, as “the



Umberto Boccioni’s bronze sculpture *Development of a Bottle in Space*, 1912, Kunsthau, Zurich — one of various casts of this work.

luminous nucleus” of scientific understanding, must be extended by “instinct” — although “instinct, even when enlarged and purified into intuition, forms only a vague nebulousness”. Boccioni’s emphasis upon “states of mind” — both in his writings and as a title for paintings — clearly signalled that more was involved than scientific logic.

Boccioni strove, in works such as *Development of a Bottle in Space*, to express a series of relativities — as far as such a thing might be possible in the intractably solid medium of bronze sculpture. We are asked to experience form, space and light as interpenetrating energies rather than as separate physical entities. The spaces occupied by the artist, the work and spectator become interactively

fused. Fixed perspective is abandoned in favour of continuously mobile viewpoints, revolving in space and transformed over time.

The *Bottle* draws space into itself, and makes its own “extension into space, palpable, systematic and plastic”.

None of this is ‘scientific’ in our circumscribed sense, but it does bear witness to Boccioni’s determination to use the artists’ special toolkit of intellect, imagination, illusion and allusion to forge his own kinds of experiment in the new age. □

Martin Kemp is in the Department of the History of Art, University of Oxford, 35 Beaumont Street, Oxford OX1 2PG, UK.

Male ticks help their mates to feed

Ixodid ticks produce a unique family of proteins that bind vertebrate immunoglobulins^{1,2}. These immunoglobulin-binding proteins (IGBPs) were discovered when it was realized that ticks excrete host immunoglobulins in their saliva during feeding³. We have sequenced three of these proteins and propose a new role for a male-specific IGBP that helps females to feed.

When blood-sucking arthropods (mosquitoes and ticks) feed, some of the host's immunoglobulins taken up in the blood-meal are not digested but pass through the gut wall and into the arthropod body⁴. This has been exploited as a route for the entry of vaccines that target 'concealed' antigens⁵. Our studies on the African tick, *Rhipicephalus appendiculatus*, revealed that these immunoglobulins are transported from the tick body cavity to the salivary glands, whence they are excreted in the tick's saliva back into the host, retaining their antibody-binding activity³. This led to the discovery of a family of IGBPs produced in the haemolymph and salivary glands of several ixodid tick species¹. Together these data indicated that IGBPs act as a 'self-defence' system against ingested immunoglobulins.

We have now characterized three abundant IGBPs specific for male *R. appendiculatus* (IGBP-MA, -MB and -MC) (GenBank accession numbers: IGBPMA, AF001868; IGBPMB, AF001869; IGBP-MC, AF001870). Sequence analysis revealed that IGBP-MB and IGBP-MC are related proteins differing in that IGBP-MC has a signal sequence, indicating that it is a secreted protein. Both proteins seem to be distant homologues of mouse and human ganglioside GM2 activator proteins⁶. The tick and mammalian proteins are of similar sizes, are secreted proteins, and bind and perhaps transport other molecules (in the case of GM2 activator, negatively charged glycosphingolipids).

IGBP-MB and IGBP-MC are stored in secretory granules of the type IV acinus, a group of specialized cells found in the salivary glands of male ticks and previously of unknown function. The male-specific IGBPs of *R. appendiculatus* are produced in abundance during the late stages of feeding², even though the male takes comparatively little blood. Adult *R. appendiculatus* were fed on guinea pigs either untreated (tick naïve control), immunized with recombinant IGBP-MC, or immunized with recombinant IGBP-MB⁷. We used immunized guinea pigs for tick feeding once they had developed specific antibodies against the respective immunogens⁷. We also placed unfed female ticks (Fig. 1a), together with male ticks pre-fed for 6 days,



Figure 1 *Rhipicephalus appendiculatus* adult ticks at various stages of feeding. **a**, Unfed female; **b**, female at 8 days of feeding; **c**, male (top) and female that were feeding in close proximity after mating, removed from a guinea pig at 6 days of co-feeding and showing a fused cement cone in the guinea pig skin; **d**, engorged female laying eggs after detachment and drop-off from the guinea pig.

on non-immune guinea pigs or on guinea pigs non-immunized but previously infested once with ticks (tick-resistant).

Female ticks showed impaired feeding on resistant guinea pigs (Fig. 2), as expected⁸. Females on animals immunized with IGBP-MC took significantly longer to feed but showed a small, although significant, increase in engorged weight, possibly resulting from the extended feeding period. In contrast, the feeding performance of females on animals immunized with IGBP-MB did not differ from the controls. Male ticks were not affected by the immunization as determined by body weight and the number of days that they survived after detaching. Females fed with pre-fed males achieved engorgement significantly earlier than the controls but still showed normal engorged weight. Female feeding might thus have been aided by the 6-day pre-fed male ticks' producing extra IGBP-MC at the initiation of female feeding.

We injected 2 µl undiluted anti-IGBP-MC serum into the haemolymph of male ticks *in situ* while they were feeding on naïve guinea pigs. Males injected with either control or immune serum died earlier than non-injected ticks after feeding, indicating that the decreased survival times resulted from inoculation trauma. However, female ticks co-fed with the anti-IGBP-MC serum-injected male ticks showed a

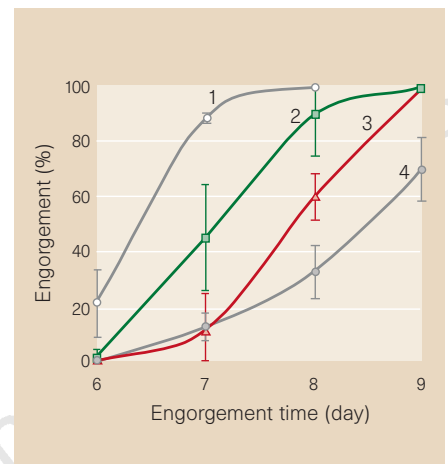


Figure 2 Comparison of feeding performance of female ticks. Female ticks were co-fed as follows: line 1, pre-fed male ticks (18 females from 2 guinea pigs); line 2, unfed male ticks (79 females, 8 guinea pigs); line 3, unfed male ticks on IGBP-MC-immunized animals (20 females, 2 guinea pigs); line 4, unfed male ticks on tick-resistant animals (30 females, 4 guinea pigs). Recombinant IGBP-MB was the full-length cDNA clone expressed as a glutathione S-transferase fusion protein in *Escherichia coli*; IGBP-MC was the full-length cDNA clone expressed with the baculovirus system.

significant decrease in body weight (24%, $F_{2,36} = 0.025$, $P < 0.05$; nested ANOVA) compared with female ticks co-fed with males injected with control serum. Engorged weights of female ticks fed with non-injected males and those fed with control-serum-injected males were similar ($F_{1,36} = 0.08$, $P > 0.05$). The time for complete engorgement was the same for female ticks in the three treatment groups.

The absence of specific detrimental effects on male *R. appendiculatus* ticks either fed on IGBP-MC-immunized guinea pigs or injected with anti-IGBP-MC serum into their haemolymph indicates that the IGBP-MC protein is not essential for male tick feeding or survival, but does affect female blood-feeding. After mating, male *R. appendiculatus* ticks reattach in close proximity to their mate (Fig. 1c). At this stage, the secretion of IGBP-MC in male tick saliva seems to affect the feeding site of the co-feeding female tick. The single enormous blood-meal of the female tick (Fig. 1b) is converted into thousands of eggs (Fig. 1d). Thus, by helping his mate to feed, the male maximizes the perpetuation of his own genes, the ultimate goal of mate guarding⁹, in this case by protecting his mate against rejection by the host. IGBPs are another example of the diverse bioactive ingredients produced by blood-sucking arthropods

that aid blood-feeding⁸. By so doing, they also promote the transmission of arthropod-borne pathogens^{10,11}.

Hui Wang, Guido C. Paesen,

Patricia A. Nuttall

NERC Institute of Virology & Environmental Microbiology,

Mansfield Road, Oxford, OX1 3SR, UK

e-mail: pan@mail.nerc-oxford.ac.uk

Alan G. Barbour

Departments of Microbiology & Molecular Genetics and Medicine, University of California, Irvine, California 92697-4025, USA

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Theropod–bird link reconsidered

Norell *et al.*¹ describe a *Velociraptor* ‘wishbone’ which they interpret as a new piece of evidence for the theropod origin of birds. The bone fits a pattern of furcula-like structures that have been discovered in certain late Cretaceous theropods, including *Oviraptor*, *Ingenia* and possibly others², some of which possess clavicles — usually not fused. But this interpretation gives rise to problems of chronology, structure and function.

First there is the temporal paradox: the earliest-known bird, *Archaeopteryx*, occurred 75–80 million years earlier than the *Velociraptor* and other late Cretaceous theropods that had ‘wishbones’. Supposed Jurassic examples are from carnosaurs, a group generally thought to be distant from birds — although the cladistic analysis of Thulborn³ places *Tyrannosaurus* closer to modern birds than to *Archaeopteryx*.

Second, the furcula-like structure in the specimen of *Velociraptor* has a round cross-section¹, while that of the primitive Mesozoic birds — *Archaeopteryx*, *Confuciusornis* and the enantiornithines — is dissimilar, being grooved postero-dorsally along almost its entire length⁴.

Also, the articulation of the arms of *Velociraptor*’s furcula-like structure along the entire margin of the coracoid (Fig. 1 of ref. 1) is unlike the articular relationship of the furcula in birds. However, it is similar to the relationship of the interclavicle bone to the coracoids found in primitive diapsids⁵. This

raises the possibility that the *Velociraptor* and theropod ‘furculae’ are nonhomologous to that of birds — the view of Bryant and Russell². Fig. 1 of ref. 1 does not show an avian articulation of the ‘wishbone’ to the rest of the shoulder girdle and resembles the restoration of Barsbold for *Oviraptor* and the mounted specimen of *Ingenia*.

Third, in most flightless birds the furcula degenerates into two clavicular splints⁶, similar to the clavicles reported in some dinosaurs². This strongly indicates that flight arose as an original function in birds, not as a modification of a structure already present before flight evolved¹.

Fourth, the presence of a furcula-like structure in *Longisquama*⁶, a primitive Triassic archosaur with long feather-like scales and postulated arboreal habits, indicates that structures of this type have developed more than once in the archosauromorphs, which means they are weak evidence for a bird link to theropods.

Alan Feduccia

*Department of Biology,
University of North Carolina, Chapel Hill,
North Carolina 27514, USA*

Larry D. Martin

*Museum of Natural History, University of Kansas,
Lawrence, Kansas 66045, USA*

Norell *et al. reply* — First, maniraptoran theropods, and theropods with furculae, are known from the Late Jurassic period^{7,8}, significantly closing the ‘gap’ discussed above. We did not say in our Scientific Correspondence¹ that *Velociraptor* was a direct ancestor, only that dromaeosaurs are related to birds. How do Feduccia and Martin explain the presence of primitive living mammals such as monotremes and marsupials 100 million years after they split from the line leading to ourselves?

Second, exact similarity is not a requirement for homology — take, for example, the forelimb variation among the living orders of mammals. Bat wings, whale flippers and human arms are very different but homologous. The furculae of living birds are highly variable⁶ and often different from the furcula of *Archaeopteryx*, yet Feduccia and Martin do not question the homology between these two groups. These authors identify this element as a possible interclavicle, a bone that is extremely different in crocodylomorphs and not present in any ornithodiran⁹.

We do not think Feduccia and Martin can, from a small photograph, say with confidence that the furcula is articulated along the entire margin of the coracoid. Examination of the actual specimen shows that the furcula attaches to the scapula just as it does in modern birds. We discussed this feature in our Scientific Correspondence¹: “...the proximal process of the furcula tapers to a point where it contacts the scapulocoracoid”.

Third, we disagree with Feduccia and

Martin’s implication that preconceived process dictates the interpretation of pattern. Science is about discovery; by assuming less, we discover more. Feduccia and Martin believe that furculae cannot exist in the non-avian Maniraptora because they did not fly, but this view precludes the possibility of furculae ever being discovered in these animals.

Fourth, *Longisquama* is a poor-quality fossil, and the interpretation of single elements is controversial. *Longisquama* lacks other characters — present in non-avian Maniraptora — that would ally it with birds. Single features do not overturn a hypothesis that is strongly supported by a plethora of character evidence.

Ironically, if one does use Feduccia and Martin’s reasoning that *Longisquama* is a close bird ‘ancestor’ as advocated elsewhere¹⁰, the temporal paradox increases. *Longisquama* comes from rocks about 220 million years old, creating a fossil-free gap of more than 80 million years before the appearance of *Archaeopteryx*. Any empirical measure of stratigraphic fit¹¹ will prefer a hypothesis of maniraptoran relationships over this one.

Mark A. Norell, Peter Makovicky

*Department of Vertebrate Paleontology,
American Museum of Natural History
New York, New York 10024, USA*

James A. Clark

*Department of Biological Sciences,
George Washington University,
Washington, DC 20052, USA*

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Height depends on month of birth

Using a large human male population of 507,125, we find clear evidence for a dependence of body height at age 18 on birth month. Over 10 years there is a sinusoidal variation with a period of 1.0 year with maxima in spring and minima in autumn differing by 0.6 cm. Although global environmental factors¹ are small and can be studied only with the help of sophisticated methods on very large sample sizes, they might offer insights into still undiscovered mechanisms of human development. This may provide empirical facts for clinical

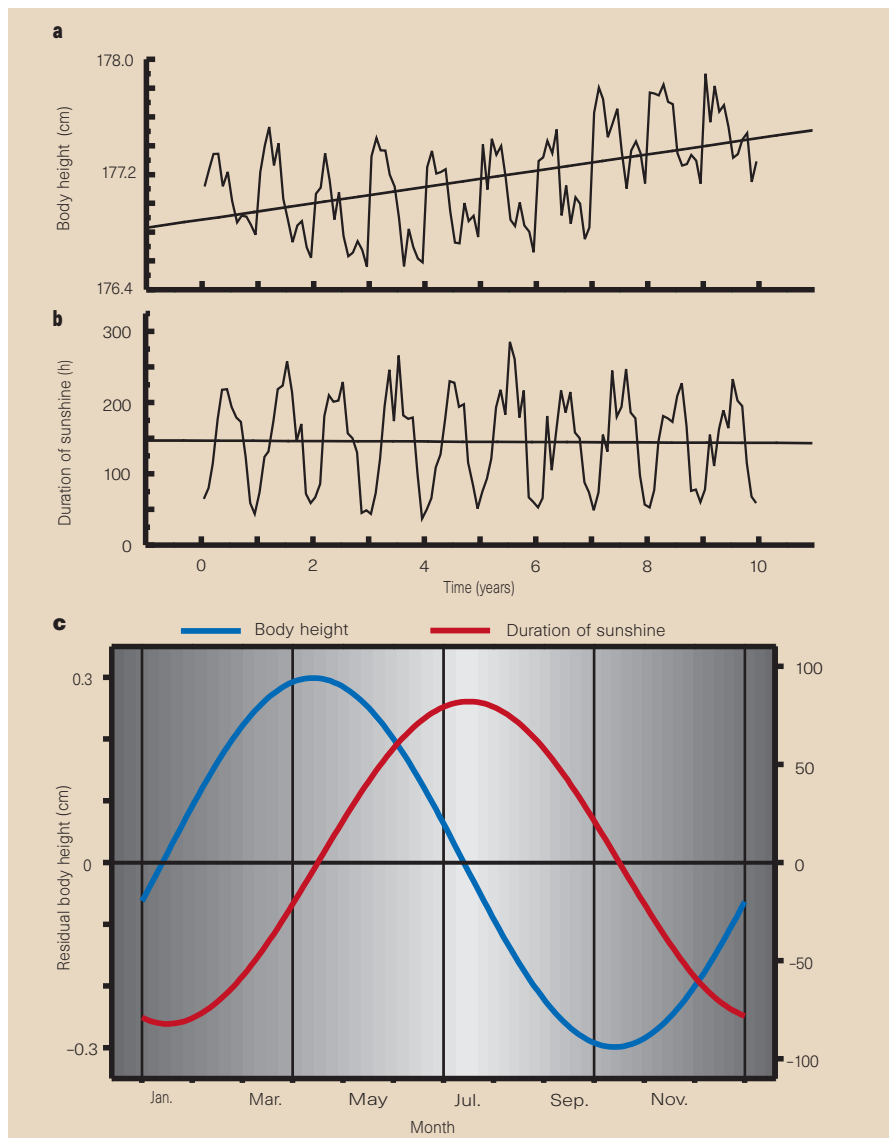


Figure 1 Relationship between body height and birth month. **a**, Monthly averages of height at age 18 (birth cohorts 1966–75). **b**, Duration of sunshine (1984–93). In both graphs, linear interpolations indicate trends¹¹. Mean and standard deviation for height, 177.2 ± 0.33 cm with 0.057 cm yr^{-1} secular trend; for sunshine duration in the same interval, 144 ± 65 h with a trend of -0.69 h yr^{-1} . **c**, Variation in height and sunshine duration residuals, as determined by the inverse Fourier transform of the signal with the largest amplitude ($P < 0.001$), during one year. The next largest amplitude ($P < 0.04$) with a period of 10 years is 0.12 cm. Maximum height is reached in April, minimum in October. Maximum duration of sunshine is in July, minimum in January.

research on the pineal gland and melatonin².

The body heights of the entire male population of ten 1-year birth cohorts of Austria were measured by the Austrian Federal Army and recorded (in millimetres and rounded to the nearest centimetre) throughout the whole year in five conscription centres. Not only is there a difference between the 'January to June' and 'July to December' cohorts (an effect that Breiting³ has already noted) but also a sinusoidal fluctuation over the year. We also compared this fluctuation with the monthly variation in sunshine duration in Austria⁴ during the same interval (Fig. 1a, b).

To demonstrate the periodic nature of the observed variations, we linearly interpolated both data sets (body height and sunshine

level) to find — and then subtract — the long-term linear trends. We analysed the resulting residuals in two different ways: by generating a Fourier spectrum and by producing a Lomb–Scargle⁵ periodogram. We found the amplitudes, the periods and the phase difference between the two data sets by Fourier analysis and tested the assumption of periodicity by calculating the Lomb–Scargle normalized periodogram, which can reveal the existence and the frequencies of periodic signals and their statistical significance in seemingly random, unevenly sampled data in the presence of some degree of (gaussian) noise. The body heights vary periodically (amplitude ± 0.3 cm) with a 365-day period, as does the duration of sunshine. There is also a constant phase shift of $+89$ (or

-276) days between the maximum body height and the maximum light level from the Sun (Fig. 1c).

We cannot offer definitive explanations of why body height depends on the month of birth with such a pronounced cycle. Yet all proposed hypotheses must consider the clear annual rhythm, which is a strong indicator of a synchronization with extraterrestrial conditions, and the nonlinear growth curves during the prenatal and early postnatal period for humans. Growth is fastest during the three months before and after birth, so we postulate that the periodicity is due to influences extending from the time of late pregnancy to the first postnatal year. Furthermore, because seasonal photic input is known to regulate both various body functions and growth in mammals, we infer a phylogenetic remnant in humans.

The underlying physiological mechanism might involve the light-dependent activity of the pineal gland. The magnitude of the melatonin concentration is related to the intensity, as well as the wavelength, of light incident on the optical apparatus. Melatonin is active during the prenatal period by transplacental passage⁶, and its cyclic production in the newborn is already established by 9–15 weeks after birth². Some authors postulate effects of melatonin on growth hormones⁷; others are convinced of an association of growth with blindness⁸.

In rhesus macaques, chimpanzees and humans, during the later part of fetal life and for a short time after birth, the arcuate nuclei in the middle hypothalamus have an important role in growth. Recent studies suggest effects of the arcuate nuclei on the growth hormone⁹ as well as the binding of melatonin in that hypothalamic structure¹⁰. Perhaps our observed periodicity in body height will provide impetus to further discussion on mechanisms of human growth.

Gerhard W. Weber, Hermann Prossinger, Horst Seidler

*Institute of Human Biology, University of Vienna
Althanstrasse 14, A1090 Vienna, Austria
e-mail: gerhard.weber@univie.ac.at*

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Rubber hands 'feel' touch that eyes see

Illusions have historically been of great use to psychology for what they can reveal about perceptual processes. We report here an illusion in which tactile sensations are referred to an alien limb. The effect reveals a three-way interaction between vision, touch and proprioception, and may supply evidence concerning the basis of bodily self-identification.

Each of ten subjects was seated with their left arm resting upon a small table. A standing screen was positioned beside the arm to hide it from the subject's view and a life-sized rubber model of a left hand and arm was placed on the table directly in front of the subject. The subject sat with eyes fixed on the artificial hand while we used two small paintbrushes to stroke the rubber hand and the subject's hidden hand, synchronising the timing of the brushing as closely as possible.

After ten minutes, subjects completed a two-part questionnaire that requested an open-ended description of their experience and asked them to affirm or deny the occurrence of nine specific perceptual effects (Fig. 1). The completed questionnaires indicated that subjects experienced an illusion in which they seemed to feel the touch not of the hidden brush but that of the viewed brush, as if the rubber hand had sensed the touch.

We hypothesized that this illusion involves a constraint-satisfaction process operating between vision, touch and proprioception — a process structured by the correlations normally holding among these modalities. Specifically, a connection-

ist model suggested that the illusion's spurious reconciliation of visual and tactile inputs relies upon a distortion of position sense.

This was tested in a second experiment by exposing subjects to the conditions of our first experiment for a prolonged period, and then probing for signs of a distortion of proprioceptive information. Both before and after the viewing period, subjects completed a series of three intermanual reaches. With eyes closed, the right index finger was drawn along a straight edge below the table until it was judged to be in alignment with the index finger of the left hand, which rested on the table in the same position as during the exposure period. Subjects' reaches after experiencing the illusion were displaced rightward toward the rubber hand, the magnitude of this displacement varying significantly in proportion to the reported duration of the illusion (Fig. 2).

A control group in which a small asynchrony was introduced between the brushing of the two hands reported low prevalence of the illusion (mean 7% of the exposure period compared to 42% for the synchronously brushed group; $P<0.01$), and failed to display reach displacement in the direction of the artificial hand (mean displacement 13 mm away from the rubber hand, compared with 23 mm towards it in the experimental group; $P<0.04$).

This illusion belongs to a class of perceptual effects involving intersensory bias¹⁻⁴. In closely related work, Ramachandran *et al.* got phantom limb patients to view their intact arm in a mirror, so that their amputated arm appeared to have been resurrected. Several subjects viewing the reflection as the intact arm was touched reported feeling the touch in the amputated (phantom) limb⁵.

Also relevant is the finding of cells in the

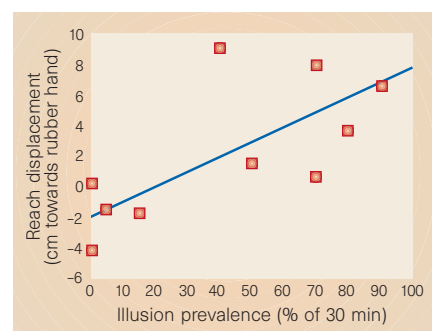


Figure 2 Results of reaching experiment. The x-axis indicates the percentage of the 30-min viewing period during which the ownership illusion was experienced. The y-axis indicates displacement of the three reaches made after the viewing period from the three made before, calculated as the difference between the means in the two groups. The data is fitted with a least-squares regression line ($y = 0.09x - 1.4$, $r^2 = 0.47$, $p < 0.03$).

premotor cortex of monkeys which respond both to tactile stimulation of a particular body region and to visual perception of an object approaching that area⁶. The connectionist network referred to above features a layer of units with analogous response properties, units that appear to be necessary for the relevant cross-modal interactions to occur.

It has been proposed that the body is distinguished from other objects as belonging to the self by its participation in specific forms of intermodal perceptual correlation^{7,8}. Subjects in our first experiment who referred their tactile sensations to the rubber hand also consistently reported, in both sections of the questionnaire, experiencing the rubber hand as belonging to themselves. Indeed, eight of ten subjects spontaneously employed terms of ownership in their free-report descriptions, for example: "I found myself looking at the dummy hand thinking it was actually my own."

While the rubber hand illusion does not tell us precisely what ingredient might make only certain forms of intermodal correlation relevant to the self, it does show that intermodal matching can be sufficient for self-attribution.

Matthew Botvinick, Jonathan Cohen
Department of Psychiatry, University of Pittsburgh, and Department of Psychology, Carnegie Mellon University, Pittsburgh, Pennsylvania 15213, USA
email: mmb@crab.psy.cmu.edu

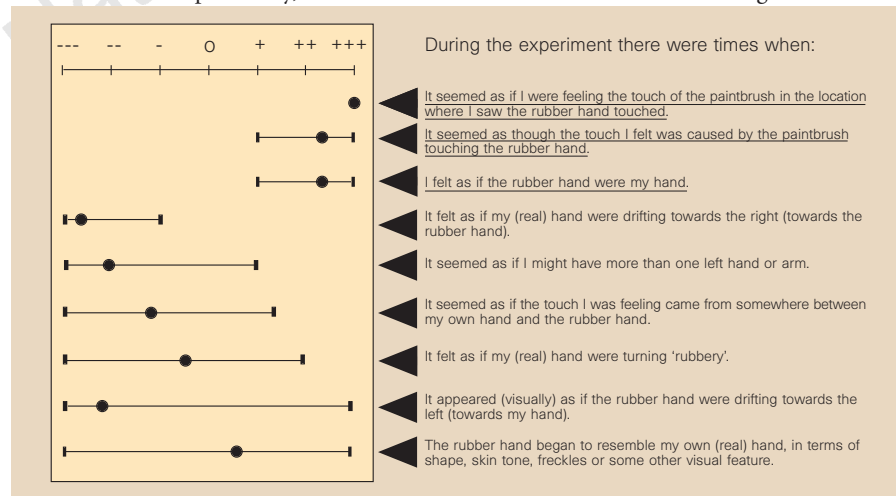


Figure 1 Questionnaire results. The questionnaire included the nine statements shown, presented in a random order. Statements describing the predicted phenomena are underlined. Subjects indicated their response on a seven-step visual-analogue scale ranging from 'agree strongly' (+++) to 'disagree strongly' (---). Points indicate mean responses. Bars indicate response range. The questions underlined showed a statistically significant tendency to evoke affirmative responses ($P<0.002$ for underlined questions, $P<0.018$ after correcting for multiple comparisons).

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Natural entertainments

Spectacular Nature: Corporate Culture and the Sea World Experience

by Susan G. Davis

University of California Press: 1997. Pp. 313.

\$18.95, £14.95 (pbk)

The Modern Ark: The History of Zoos – Past, Present and Future

by Vicki Croke

Scribner: 1997. Pp. 254. \$26

Stephen Bostock

You might wonder why a sociologist at San Diego University should devote several years to fieldwork in her local 'theme park', Sea World, and whether her results would be of interest to non-sociologists. I wondered too for a page or two at the start of *Spectacular Nature*—I recoil from jargon such as "public space" and "integrated landscape of meanings"—but Susan Davis had won me over before the end of her introduction. I was soon in a state of eager anticipation to learn more, and I was not disappointed.

Davis is particularly interested in establishing and examining the message Sea World is conveying. Obviously, if it is a pleasure park pure and simple, then its message (if any) and activities concern only its customers, except, say, for social historians, and animal welfarists and 'rightists' concerned about capturing, keeping and training killer whales and dolphins. But Sea World, although it does provide entertainment with the utmost success to three million or more visitors each year, also claims a higher role, that of educating its customers into an appreciation of wildlife and instructing them in how to help conserve it. The public are even told that they are helping the cause of conservation by merely coming to Sea World! So to study what exactly Sea World is communicating is clearly a matter of interest and importance.

Davis is interested in Sea World also as a human phenomenon, a complex and highly organized institution, and the multiplicity of angles from which she examines it makes for a richly informative book. For example, as a social historian as well as a sociologist she puts Sea World into its historical context. She examines other ways in which nature has been represented, and relates Sea World to the entertainment industry, and not least, where appropriate, to its ancestry in the circus world. All the different strands of her investigation are supported by lively, although unobtrusive, notes and a multitude of references.

The amount of intriguing information she provides is astonishing, such as the marvellously detailed account of how Sea World monitors its visitors, checking their numbers



Out for the count: Sea World keeps exact records of every training session and performance.

hourly against exact forecasts to ensure the level of income required by Sea World's stern and money-minded owners, Anheuser-Busch, the world's largest brewer. Sea World's extreme attention to detail is evident in another department, that of the animal trainers, who keep exact records of every training session and every performance (of which there may be up to six daily) of every killer whale or dolphin, and presumably of every sea-lion and otter too. This is necessary because every trained animal must be treated as an individual.

Interestingly, what Davis convincingly portrays as a sort of total control that Sea World seeks to exercise (quite successfully) over every employee and activity, and even in a way over the public (for example, music at every exhibit indicates the 'correct' emotional response), breaks down a bit with the cetaceans. Their interest and attention must be maintained, or they will stop cooperating. So there is essential flexibility in (especially) the whale shows, and occasional disobedience by the animal performers, sometimes actual and sometimes (as in traditional circuses) rigged for humorous effect. The

training is mainly by operant conditioning with positive reinforcement only: they are rewarded, but are not punished for failure. But many will still feel that cetaceans should not be trained, or kept at all.

Sea World has evolved rapidly in its 30 or so years, often, Davis convincingly argues, as a result of outside pressures, not least perhaps animal rights demonstrations. One response has been increasingly to claim a serious educational and conservational role, indeed to give every impression of being a public institution. However motivated by business interests such responses are, they may still surely be worthwhile in themselves, an example being the setting up of Sea World's scientific partner, Hubbs-Sea World Research Institute.

But Davis shows Sea World's conservation education to be absurdly limited: individuals are exhorted to avoid polluting or otherwise harming the environment of the marvellous sea creatures they gaze upon at Sea World. But no word is ever breathed that industry might damage the environment; there can be no suggestion that Anheuser-Busch (or its like, presumably) is other than a

JERRY ROBERTS/FRANK SPOONER PICTURES

faithful guardian of the natural world. Mention of evolution is now banned too, as presumably too controversial, and this in an institution that seeks increasingly not only to encourage school parties but also to provide biological instruction to schools on a vast scale through television and the Internet. There are important issues here, on which Davis has shone a remarkable light. Even to persuade such an institution to let her interview its staff — without editorial control — is no mean achievement.

Vicki Croke's account of the history of zoos in *The Modern Ark* is a less original study and on an altogether smaller scale. But it is superbly written, with an enviable lightness of touch, which is just right for expressing the magic of the best in zoos and the opportunities they sometimes offer for real appreciation of their animals, such as gorillas at Chicago's Lincoln Park or a fur-seal watched (and watching back) through an underwater viewing panel at Tacoma, Washington. Some of Sea World's exhibits, such as a splendid Antarctic simulation for penguins, must also belong in this category (apart, for me, from the music). Croke's gorilla and seal examples come near the start of her book, partly as a backcloth for her recognition of zoos' all too frequent failure to live up to their own aspirations.

Hers is thus a fair, even-handed account. And it is a well informed one, especially regarding new developments in zoos, in the United States in particular, and her account of the earlier history of zoos is refreshingly lively. Particularly useful are her wise discussions of such controversies as the pros and cons of keeping white tigers (or even white lions) and the appalling problem of whether zoos are justified, even for high conservation motives, in culling so-called surplus animals. □

Stephen Bostock is at Glasgow Zoo, Uddingston, Glasgow G71 7RZ, UK.

Money spinner

Fractals and Scaling in Finance

by Benoit B. Mandelbrot

Springer: 1997. Pp. 456. \$39.95, £32.50

Ian Stewart

In the late 1950s and early 1960s, Benoit Mandelbrot, then a young and relatively unknown researcher at IBM's T. J. Watson Research Center, devoted a lot of his time to problems in economics and finance. His ideas in these areas formed a tiny part of a huge body of work, ranging from rainfall statistics to linguistics, that led him to create the concept of the fractal, for which he is now famous all over the world.

Fractals and Scaling in Finance brings Mandelbrot's work full circle, applying

today's mature fractal geometry to the problems that plagued him 40 years ago. It is a typically Mandelbrotian mix of reprinted papers, commentary, unpublished results and new work hot off the press. Its focus is simple: what is the structure — if any — of financial data? Its scope is so broad that I cannot hope to summarize it adequately.

The world's stockmarkets have recently been in turmoil as the FTSE 100 Index and the Dow Jones plunged headlong after the Nikkei and the Hang Seng. Some weeks the market has a clear dynamic, and it is down; but sometimes it is up. Who knows which it will be next week? Faced with such unpredictability, classical mathematics took one look at the financial world, deemed it to be random, and the paradigm was set. The great triumph of modern financial mathematics — the famous Black–Scholes equation of 1973, without which there would be no derivatives market — is based on a purely stochastic model.

Just as biologists 'know' that evolution is random — despite a wealth of evidence to the contrary — so financial analysts 'know' that the market is random. The biologists' mistake is to confuse a dynamic with a goal. Evolution may well have no predetermined goals, but it manifestly has an emergent dynamic. Emergent phenomena are easily confused with random ones, because neither has an accessible 'audit trail' of cause and effect. In random phenomena — or more accurately, in models of them — there is no audit trail, whereas in an emergent phenomenon it exists but is too complicated to describe.

Financial analysts have made the same mistake. The market has all the characteristics of an emergent system, and the evidence that market data possess hidden structure is becoming overwhelming. Measures of the degree of order in a time series, such as the 'approximate entropy' introduced by Steve Pincus, reveal that the markets generally remain well below the maximum level of disorder. The only time the Dow Jones index came close to maximum disorder was immediately before the stockmarket crash of 1987.

But what kind of structure do market data possess? According to Mandelbrot, their central feature is scaling. Roughly speaking, the small-scale fluctuations of the market mimic the large-scale ones, but on a compressed timescale. The genesis of this principle was Mandelbrot's early realization that orthodox statistics can tell us very little about financial data. The reason, he believed, is that for financial data the standard deviation is infinite. This implies, for example, that jumps in market value can be bigger, and more rapid, than conventional statistics would allow.

The simplest statistical distributions

with this feature are power-law distributions, a far cry from the beloved (and dreadfully overrated) Gaussian of the textbooks. In a way, the entire book is the story of Mandelbrot's intellectual voyage into power-law territory, as he refined his early observations into more sophisticated, but always simple and elegant, models of the irregularities of financial time series.

The high point is the theory of L-stable processes and their generalizations, which capture many characteristics of market data in both qualitative and quantitative ways. It would be interesting to relate these ideas to recent work that models markets as 'adaptive complex systems', which are collections of entities (traders) interacting according to simple rules, from which the overall market emerges. There is growing evidence that such systems capture many features of financial markets, and may even be a way of finding that elusive element of 'structure'. Are there credible micro-rules that generate macro L-stable statistics?

This is not an easy book to read, but once one gets into the flow of ideas it is rewarding and full of insight, and should be on every chartist's bookshelf. There is one weakness, though, that will be apparent to any child of the computer age: a neglect of modern data. Mandelbrot explains that, although extensive data are now available, he is "ill equipped for empirical work". Perhaps... but isn't that what postdocs are for? □

Ian Stewart is in the Department of Mathematics, University of Warwick, Coventry CV4 7AL, UK.

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A weighty problem

The Fat of the Land: The Obesity Epidemic and How Overweight Americans Can Help Themselves

by Michael Fumento
Viking: 1997. Pp. 330. \$25.95

John Wilding

The epidemic of obesity imposes a heavy burden on the healthcare systems of many industrialized countries, contributing up to 10% of total health costs. The United States leads the way, with a third of the population classified as obese, but Europe is catching up rapidly. Why? What should be done to stem the rise, and how should we treat those who are already obese?

Michael Fumento is a medical journalist who, frustrated by his own repeated cycles of weight loss and regain, has immersed himself in the medical, scientific and general literature on obesity. He learnt the facts about the obesity epidemic: its probable causes, the risks obesity poses for the individual, and the many claims for cures, ranging from the latest diets and drugs to outrageous quackery. Finally, he managed to lose weight and keep it off. The book is a summary of this literature, interspersed with personal observations and anecdotes and his own (sometimes controversial, and often not politically correct) solutions to the problem.

Perception of risk, as Fumento recognizes, is one of the main barriers to the health hazards of obesity being universally accepted; people worry more about alar on apples, radon gas or BSE than about obesity, yet it kills 300,000 Americans a year. They are confused by the mixed messages coming from the media, advertisers, the food industry, 'experts' in the slimming industry and the 'fat acceptance' lobby, each with their own agendas and vested interests. Fumento sees the success of 'miracle' diet books, products that claim they cause fat to 'melt away', or electrical pads that burn calories by 'stimulating minute muscle contractions' as a symptom of people's willingness to believe in anything that allows them to lose weight without changing their behaviour.

Why is obesity increasing? The population is eating more and exercising less. Labour-saving devices, especially cars, and the food industry are the usual suspects, and plenty of evidence is given to convict them. Low-fat foods and artificial sweeteners are discussed and seen as an excuse to eat other high-calorie foods rather than as a solution.

Unfortunately, some important scientific issues are dismissed or glossed over. Genetic differences are clearly not the cause of the current epidemic of obesity, but they



Gone to waist? Perception of risk is one of the main barriers to the health hazard of obesity being universally accepted.

may nevertheless be important. Twin studies have shown that the tendency to gain weight in response to overfeeding is largely inherited. Some people gain weight more easily than others and find it harder to lose, which may partly explain why diet and exercise do not work equally well for everybody. And there is no mention of the hypothesis proposed by Barker and Hales that suggests that smaller babies are more prone to many of the medical problems associated with obesity, such as diabetes, hypertension and heart disease, because of fetal metabolic programming. The current demographic bulge in the adverse effects of obesity (and possibly in the tendency to gain weight when food is plentiful) may therefore lessen in future generations as the nutritional state of mothers improves.

The solutions proposed and advice given by Fumento are generally sound. Yet teaching people the basic principles of energetics and then persuading them to eat less and exercise more may not be enough. Even with the best behavioural programmes, only a small percentage of people maintain their weight loss in the long term. These successful dieters and exercisers (such as Fumento himself) are rightly highlighted, and I would agree that more work needs to be done to discover why these people succeed while most do not.

Fumento understands that obesity is a serious medical problem, yet he is wary of medical solutions. Since the book was published, the appetite-suppressant drugs

fenfluramine and dexfenfluramine have been withdrawn because of reports of their involvement in valvular heart disease, particularly when taken in combination with phentermine. Some new drugs are discussed, such as the intestinal lipase inhibitor orlistat, which reduces the absorption of fat, but sibutramine, a serotonin and noradrenaline reuptake inhibitor, is not mentioned.

Despite Fumento's scepticism, the rapid development of scientific and commercial interest in obesity may yet uncover new ways to help weight loss; I feel it is too early to dismiss a pharmacological solution. Strangely, surgical treatments are not mentioned at all, although they produce the most dramatic weight loss and provide the best evidence that losing weight is beneficial to health.

I enjoyed this book, which is a refreshing alternative to other popular books that discuss weight and how to lose it. It is amusingly written, but the humour at the expense of the obese may alienate those it seeks to help. Fumento sees this humour as a tool for a change in society, like the effect peer pressure has had on smoking in some groups. But perhaps it is the environment we have created that is at fault, not the individual. I agree that obese people must take on the responsibility of changing themselves, but there is a fine line between persuasion and persecution. □

John Wilding is at Fazakerley Hospital, Longmoor Lane, Liverpool L9 7AL, UK.

In retrospect chosen by David Ruelle

Science et Méthode

by Henri Poincaré
(1908)

Henri and Raymond Poincaré were a gifted pair of cousins. Raymond, born in 1860, would become president of the French Republic. Henri, born in 1854, was to be a famous mathematician, and he is the one whom scientists simply call Poincaré. Young Henri had varied interests, and spent his spare moments writing a novel: the story of a heartbroken beautiful young woman, in an aristocratic setting. The unfinished manuscript of the novel now seems to be lost and, judging by a few extant fragments, this may be just as well.

Henri Poincaré's masterpiece would come later: the three volumes of *Les méthodes nouvelles de la mécanique céleste* (1892–99) were a different kind of literature. In this opus, and a deluge of articles and books, Poincaré opened up or revolutionized vast areas of mathematics: topology, the theory of algebraic curves, dynamical systems and so on. He was a powerful mathematician, with universal interests extending also to physics. His intellectual vigour remained unaltered right until his death in 1912 from complications of a prostate operation.

To be confronted with an intelligence of this magnitude is a bit disturbing. Should we exclaim that such genius is beyond comprehension? Or should we look for weak points, as French mathematicians of a later generation have done, finding Poincaré's methods too intuitive and his interests outmoded? Or perhaps we should approach the general problem of how a mathematician's brain works.

This problem, characteristically, was one that Poincaré himself pondered. He knew, as every mathematician does, that if you have to solve a difficult problem, you first spend time looking at it from different angles. A number of ideas present themselves, which you pursue conscientiously, but you fail to solve your problem. How then do you proceed? Here is what may happen: "One evening I took black coffee, contrary to my custom. I could not go to sleep. Ideas came up in swarms, I sensed them clashing until a pair would hook together, so to say, to form a stable combination. By morning... I had just to write the results, which only took me a few hours."

Poincaré's hooking together of ideas may take place unconsciously. He describes how he had at some point to abandon his mathematical preoccupations to go on a geological trip and "... we took a bus for some excursion or other; the instant I set my foot on the step the idea came to me, with nothing apparently in my previous thoughts having prepared me for it, that the transformations I had used to define Fuchsian functions were



Henri Poincaré: powerful mathematician with universal interests.

identical with those of non-Euclidean geometry. I did not make the verification; I should not have had the time... but I instantly felt a complete certainty."

Reflecting on several such experiences, Poincaré expresses his belief that what appears to be a sudden illumination is in fact the result of previous long subconscious work. Some card-carrying rationalists might frown at the role attributed by Poincaré to unconscious thought in a higher intellectual process. Poincaré was actually a very rational man: here he presents some interesting facts of observation, and boldly discusses their implications. But, characteristically, he stops when he reaches the end of what he can seriously argue.

The above quotations come from chapter 3 of *Science et méthode* (1908). This is one of several books in which Poincaré presented his ideas on the philosophy of science to the general public. Young Henri had failed in his early attempt at a heart-stirring novel, but there is no doubt that he had a literary gift. The style of his philosophical writings is fluid and unhurried, and a delight to read. The clarity of exposition is such that you forget about the style and concentrate on the ideas. Poincaré's books on the philosophy of science consist of short chapters, each a little essay. Some chapters on physics are out of date, but others have aged well, and sometimes show uncanny premonition of future scientific developments.

Chapter 4 is about chance, or randomness. Where does it come from? Poincaré finds not one but several sources of randomness (the list would be longer now with the advent of quantum mechanics). He explains for instance that randomness in roulette arises from our lack of muscular control in spinning the wheel. Another source is what we now call chaos: "A very small cause, which escapes us, determines a

considerable effect, which we cannot ignore, and we then say that this effect is due to chance." As an example he discusses meteorology: "Why do rainstorms... seem to happen at random, so that many people find it natural to pray for rain or fair weather, when they would judge ridiculous to ask for an eclipse?" The answer he gives, sensitivity to initial conditions, would be rediscovered (and justified) much later.

The elegant simplicity of Poincaré's presentation hides the vast amount of mathematical expertise and thinking underlying his philosophical discussions. And all this thinking did not always reach a satisfactory conclusion. It is probable for instance that he spent time thinking about hydrodynamic turbulence. He wrote a book related to the topic (*Théorie des tourbillons*, 1893), a study of vortices and their stability, but does not obtain any dramatic result.

One can guess that Poincaré's intelligence has a significant moral aspect. Because he was basically modest, and his work had received considerable recognition, he showed little of the bitterness and need to assert superiority that are common traits among great (and less great) scientists. He was not a militant person: he said of the rather more ideological Kronecker that the latter could do so much fine mathematics because he frequently forgot his own mathematical philosophy. Poincaré was thus not driven by strong ambition or ideological preconceptions, but had an extreme curiosity for the true nature of things. And so we see him pushing his investigations often far ahead of his time, unafraid to venture into physics and psychology, but stopping short of poetical guesswork, which seems to have held no attraction for him.

David Ruelle is at the Institut des Hautes Etudes Scientifiques, 91440 Bures sur Yvette, France.

Dax1 antagonizes Sry action in mammalian sex determination

Amanda Swain*, Veronica Narvaez*†, Paul Burgoyne*, Giovanna Camerino‡ & Robin Lovell-Badge*

* Division of Developmental Genetics, MRC National Institute for Medical Research, The Ridgeway, Mill Hill, London NW7 1AA, UK

† Facultad de Ciencias, Universidad Autonoma del Estado de Morelos, Av. Universidad 1001, Col. Chamilpa, CP 62210, Cuernavaca, Morelos, Mexico

‡ Biologia Generale e Genetica Medica, Universita di Pavia, via Forlanini 14, 27100 Pavia, Italy

DAX1, which encodes an unusual member of the nuclear hormone-receptor superfamily, is a gene that may be responsible for a sex-reversal syndrome in humans, referred to as dosage-sensitive sex reversal, in which XY individuals carrying duplications of Xp21, part of the small arm of the X chromosome, develop as females. XY mice carrying extra copies of mouse Dax1 as a transgene show delayed testis development when the gene is expressed at high levels, but do not normally show sex reversal. Complete sex reversal occurs, however, when the transgene is tested against weak alleles of the sex-determining Y-chromosome gene Sry. These results show that DAX1 is largely, if not solely, responsible for dosage-sensitive sex reversal and provide a model for early events in mammalian sex determination, when precise levels and timing of gene expression are critical.

Sex determination in mammals depends on the Y-chromosome gene *Sry*, which acts within the developing gonads to establish a programme leading to differentiation of testes rather than ovaries¹⁻³. Several pieces of evidence show that *Sry* functions in a cell-autonomous manner to trigger the differentiation of Sertoli cells from one of the somatic cell lineages in the indifferent gonad (genital ridge)⁴⁻⁷. In the absence of *Sry*, or of its functional protein product (SRY), it is thought that the cells of this 'supporting cell precursor' lineage differentiate into follicle (granulosa) cells. The question of how sex determination operates in mammals can therefore be reduced to a decision of cell fate: do Sertoli cells form or not. Once Sertoli cells begin to differentiate they must then signal to the other cell lineages that they should follow the male pathway, leading to the formation of a functional testis.

In the mouse, expression of *Sry* occurs only briefly, from 10.5 days *post coitum* (d.p.c.) to 12.0 d.p.c. (refs 4, 5). As Sertoli cells are not recognizable until after this time, it follows that SRY must influence the activity of other genes which subsequently ensure the differentiation and maintenance of Sertoli cell characteristics. The function of SRY is largely ascribed to an HMG-box DNA-binding domain, which is the only conserved part of the protein⁸⁻¹¹. On binding DNA, this domain induces a sharp bend in specific DNA sequences and it is thought that SRY acts as an organizer of local chromatin structure¹². It is not clear, however, whether it participates in the activation of secondary testis-determining genes, the repression of ovary-determining genes or in both processes. It will be necessary to define target genes, but it is also imperative to understand the mechanisms that lead to ovary differentiation.

The first morphological changes to distinguish an ovary from the indifferent gonad are not apparent until several days after overt testis differentiation has occurred¹³. Germ cells begin to enter meiosis from 13.5 d.p.c., but follicles do not form until several days later. Nevertheless, ovary differentiation is unlikely to be passive as there are changes in gene expression that occur very early in XX genital ridge development. For example, *Sox9* is expressed at a low level in the genital ridges of both sexes, but, coincident with its upregulation in the XY genital ridge (following the onset of *Sry* expression), it is turned off in the developing ovary^{14,15}. This may be due to the action of a repressor, which could be considered to function as an anti-testis or ovarian determinant.

Loss-of-function mutations in an ovary-determining gene should not affect male development of XY embryos, but gain-of-function mutations might be expected to give sex reversal and XY female development. One clear case of where this happens in humans is

known. XY individuals with a duplication of part of the short arm of the X chromosome and an intact *SRY* gene show male-to-female sex reversal. The single X chromosome in these individuals does not undergo X-chromosome inactivation: therefore, these individuals presumably carry two active copies of genes in the duplicated region. The minimum duplication responsible for this syndrome, referred to here as dosage-sensitive sex reversal (DSS), has been mapped to a 160-kilobase (kb) region of Xp21 (ref. 16). Critically, XY individuals with deletions of the entire DSS region develop as males¹⁶. Genes within the DSS region are therefore not essential for testis development, but, when present in a double dose, at least one of them can interfere with testis formation. It follows that this gene may have an important role in ovary development¹⁶.

Several genes map to the 160-kb minimum duplicated region in DSS patients. These include the *DAM* genes, which constitute a family of four to five genes related to the *MAGE* family that encode tumour-associated antigens but whose function remains obscure¹⁷. The *DAX1* gene, however, which encodes an unusual member of the nuclear hormone receptor superfamily, has been considered most likely to be responsible for DSS. Mutations within *DAX1* lead to adrenal hypoplasia congenita (AHC), a syndrome also found in XY patients deleted for the DSS region^{18,19}. These patients show defects in adrenal cortex function and hypogonadotropic hypogonadism. Cells of the adrenal cortex originate from the genital ridge, indeed, expression of the mouse *Dax1* gene is associated with early genital ridge development and with the adrenal, pituitary and hypothalamus²⁰⁻²². *Dax1* begins to be expressed at the same time as *Sry*, but in the genital ridge of both XX and XY embryos. As testis development proceeds it is rapidly downregulated, whereas it persists throughout ovary development. This suggests that *Dax1* could be involved in sex determination where its prolonged expression is incompatible with testis development²⁰.

We have therefore used transgenic mice to see whether *DAX1* could be responsible for DSS. Specifically, we set out to test the ability of extra copies of the *Dax1* gene alone to induce female sex reversal of XY mice.

Expression of early genital ridge markers

If *Dax1* interferes with *Sry* action, the two genes should be expressed in the same cells at the same time. Figure 1 shows whole-mount *in situ* hybridization for *Sry*, *Dax1* and *Sox9* transcripts in genital ridges at 11.5 and 12.5 d.p.c. We also included anti-Müllerian hormone (*Amh*) gene expression as an early Sertoli cell specific marker^{4,23,24}. The *Sry*, *Dax1* and *Sox9* transcripts show a broadly

similar distribution in XY genital ridges at 11.5 d.p.c. *Sry* expression is much weaker than that of the other two genes and shows a gradient with highest levels in the posterior region of the gonad. This may reflect a wave of expression passing down the genital ridge, as the signal is stronger more anteriorly at earlier time points (data not shown). In XX genital ridges at 11.5 d.p.c., *Dax1* expression is not affected by the lack of *Sry*, but *Sox9* expression has been extinguished. One day later, *Sry* transcripts are no longer detectable in XY gonads (not shown) and *Dax1* shows a reciprocal expression pattern when compared with *Sox9* and *Amh*, with *Sox9* and *Amh* expressed at high levels in differentiating Sertoli cells in the testis whereas *Dax1* expression is maintained in the ovary.

Genital-ridge-specific *Dax1* regulatory region

To define the regulatory elements of the *Dax1* gene, fragments of mouse *Dax1* genomic DNA were inserted upstream of the *lacZ* gene and used to generate transgenic mice. Details of this analysis will be presented elsewhere, but an 11-kb fragment upstream of the *Dax1* start of transcription (in a construct named *Dax:lacZ*) consistently showed *lacZ* expression in the developing gonad in a pattern identical to that of endogenous *Dax1* expression (Fig. 2A). Levels varied between transgenic lines, with little ectopic expression seen except for some weak staining in the posterior neural tube (not shown). β -Galactosidase activity was absent from other sites of endogenous *Dax1* expression, such as the developing adrenal gland, hypothalamus and pituitary. This 11-kb genomic fragment therefore constitutes a genital ridge/gonad-specific regulatory region of *Dax1*.

Within the genital ridge, β -galactosidase activity was first observed at about 10.5 d.p.c. and reached a high level by 11.5 d.p.c. in both male and female embryos. This activity persisted throughout ovary formation, but in the male it was rapidly down-regulated at ~12.5 d.p.c., although it was maintained in the region between the gonad and mesonephros, as is the endogenous gene²⁰ (Fig. 1). In the adult, *lacZ* expression was seen in the stroma and some granulosa cells of the ovary, and in Leydig and some Sertoli cells of the testis, again consistent with endogenous expression^{20,25}.

Although β -galactosidase activity was lost during early stages of testis development, this occurred with a slight delay in comparison to endogenous *Dax1* transcripts, such that residual activity was still seen in a few cells at 12.5 d.p.c. *LacZ* is not always a perfect reporter because β -galactosidase activity will persist for some time in cells after transcription of a transgene is downregulated²⁶. This persistent β -galactosidase activity can be advantageous, however, as it is effectively a short-term lineage marker. Analysis of sections through the whole-mounts revealed *lacZ*⁺ cells within testicular cords with typical Sertoli cell morphology (Fig. 2B). This shows that the transgene, and, by inference, the endogenous *Dax1* gene, is expressed within cells of the indifferent genital ridge that are destined to become Sertoli cells in a male. These are the same cells in which *Sry* acts.

Functional assay for *Dax1* regulatory region

If *Dax1* and *Sry* are expressed in the same cells of the 11.5 d.p.c. XY genital ridge, using the *Dax1* regulatory sequences to target *Sry* expression to XX embryos should result in a switch from ovarian to testicular differentiation. To test this hypothesis, 5 kb of mouse genomic *Sry* DNA containing the open reading frame and 3' untranslated region (UTR) was inserted downstream of the 11-kb *Dax1* 5' regulatory region and this construct (*Dax:Sry*) was used to make transgenic mice. Four out of six independent liveborn transgenic XX animals developed as phenotypically normal looking males. This frequency of sex reversal correlated with the frequency of β -galactosidase activity observed in embryos transgenic for *Dax:lacZ* (11 out of 17 animals showed β -galactosidase activity). As expected, these males were infertile and had testes that lacked germ cells because of the presence of two X chromosomes and

absence of Y-linked genes required for spermatogenesis. They appear to be identical to XX males transgenic for *Sry* under its own promoter^{27,28}.

Four founder XY males were obtained, which have transmitted the *Dax:Sry* transgene to their offspring. Sex reversal has not been seen in two of the lines, presumably because of a lack of transgene expression. All the XX transgenic descendants from one founder (D:S23) develop as males, whereas those from founder D:S8 can be male or female. Line D:S23 was therefore used to investigate the sex reversal at different embryonic stages. In addition to gonad morphology, whole-mount *in situ* hybridization was used on carefully staged embryos to look for *Amh* gene transcripts as an early marker of Sertoli cell differentiation. A delay in the appearance of testicular cords and *Amh* expression was observed for the XX transgenic gonads when compared with either non-transgenic XY testes or testes of XX embryos transgenic for *Sry* driven by its own promoter (Fig. 3). This delay corresponds to only a few hours and by 13.5 d.p.c. the morphology and *Amh* expression appear almost indistinguishable from those of normal XY testes (Fig. 3c and f).

Effect of extra copies of the *Dax1* gene

To determine whether *Dax1* can give a sex-reversal phenotype in mice equivalent to that seen in humans duplicated for the DSS region, we generated transgenic mice carrying extra copies of the mouse *Dax1* gene. For this, the *Dax1* cDNA plus the rabbit β -globin

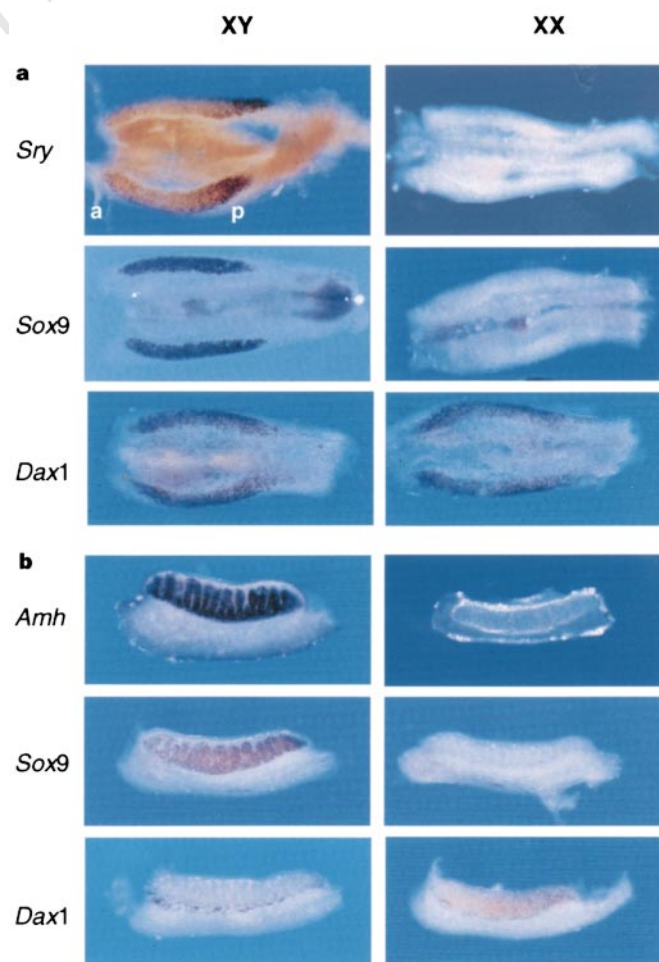


Figure 1 Expression of genital-ridge markers. Whole-mount *in situ* hybridization (shown as darker areas) on normal male (XY) and female (XX) dissected mouse gonads and underlying mesonephros is shown. Anterior (a) and posterior (p) are indicated. The genes probed in each case are indicated at the left. **a**, Gonads from 11.5-d.p.c. embryos. **b**, Gonads from 12.5-d.p.c. embryos.

intron and polyadenylation signal were inserted downstream of the 11-kb *Dax1* genital-ridge-specific regulatory region, to give the construct *Dax:Dax*. A total of 15 liveborn (F_0) transgenics was obtained, 10 males and 5 females. All the males were XY and all the females were XX. Nine of the XY males and all of the XX females transmitted the transgene to offspring. No sex reversal was apparent amongst any of these F_1 animals or their descendants. In addition, 13 transgenic F_0 embryos resulting directly from microinjection were analysed at 14.5–16.5 d.p.c. There were seven phenotypically normal XY males and six normal XX females.

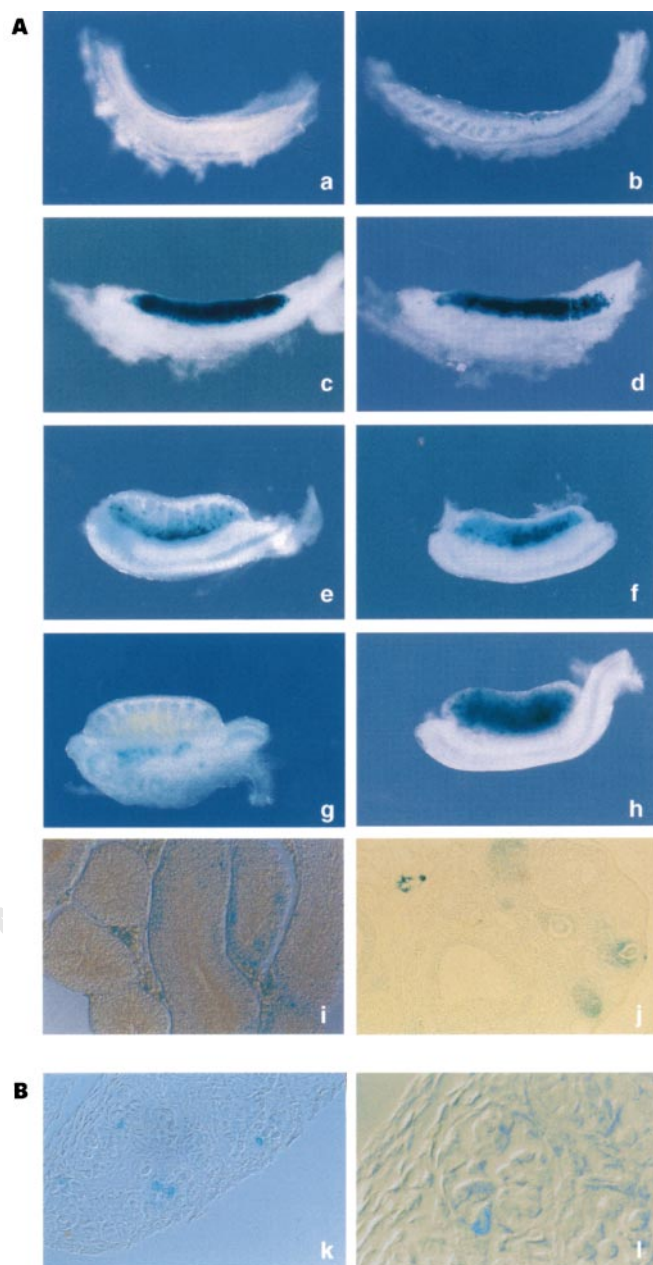


Figure 2 Expression of the *Dax:lacZ* transgene in gonad development. **A**, Transgenic XY (**a, c, e, g**) and XX (**b, d, f, h**) mouse gonads and underlying mesonephros were dissected from 10.5 (**a, b**), 11.5 (**c, d**), 12.5 (**e, f**) and 13.5 (**g, h**) d.p.c. embryos and stained for β -galactosidase activity. **i**, Frozen section from a transgenic adult testis stained for β -galactosidase activity. **j**, Paraffin wax sections from a transgenic adult ovary stained for β -galactosidase activity. **B**, Section through a 12.5-d.p.c. transgenic XY gonad shows β -galactosidase activity within testicular cords (**l** shows a higher magnification of a region in the top left-hand corner of **k**).

In addition to the possibility that *Dax1* is not responsible for DSS, there are several reasons why the mouse *Dax1* transgene might not give XY female sex reversal in mice. The most obvious is that levels of transgene expression are insufficient to achieve a double dose of DAX1 activity. The relative amounts of transgenic and endogenous *Dax1* expression in 11.5-d.p.c. urogenital ridges were therefore determined by RNase protection assays (Fig. 4). As expected from our analysis of *Dax:lacZ* and *Dax:Sry* transgenics, there was a wide range in the levels of exogenous *Dax1* expression. Many animals had very low levels, but four lines out of the ten analysed expressed at levels higher than the endogenous gene, with line 1812 being the highest with five times the normal level. Western blots, using a monoclonal antibody to DAX1 (ref. 25), of protein derived from 11.5-d.p.c. urogenital ridges from lines 1812 and 1979 confirmed that there was a corresponding increase in DAX1 protein levels (data not shown). These results showed that at least some of the *Dax:Dax* transgenic animals had total levels of *Dax1* expression that were significantly higher than the double dose expected in patients with Xp21 duplications.

Scoring only adult sexual phenotype can be misleading, as ovotestes can resolve during foetal development to either ovaries or testes and usually to the latter. We therefore looked at foetal stages of a number of *Dax:Dax* transgenic lines. Testis cord formation and *Amh* expression were found to be significantly retarded in line 1812, which expresses the highest levels of transgenic *Dax1* (Fig. 5a), but both properties were seen to recover by 13.5 d.p.c. (not shown). Marked effects were not seen in lines that expressed lower levels of transgenic *Dax1*, although testicular cords were found to be disorganized in XY embryos of line 2182 (Fig. 5a).

To increase the level of *Dax1* expression even further, hemizygous animals from transgenic line 1812 were mated together to give homozygotes. The XY animals developed as males but all three adults tested were infertile. The testes of the homozygotes were small compared to wild type (for example, some testes were 31 mg compared with a normal weight of 80 mg) and sections revealed a spermatogenic block (Fig. 5b). This phenotype could indicate that the testes were derived from embryonic ovotestes, but it could also result from postnatal overexpression of *Dax1*.

Effect of weakening *Sry*

Poschiavinus *Sry*. Our studies of the *Dax:Dax* construct indicate that *Dax1* may be mildly antagonistic to *Sry* when expressed at high levels in transgenic mice. If this is the case, the *Dax:Dax* transgene should have a more pronounced effect if it tested against weaker alleles of *Sry*. To test this hypothesis, females from the *Dax:Dax* line 1812 were bred with males carrying a *Mus domesticus poschiavinus* Y chromosome (Y^{POS}). Embryos with the Y^{POS} chromosome on a C57BL/6 genetic background invariably show sex reversal to give either XY females or hermaphrodites, although some of the latter appear phenotypically male by birth²⁹. On an outbred or mixed genetic background, Y^{POS} gives a delay in testis cord formation of about 14 h, but normal fertile males result³⁰. The delay is probably due to lower levels of expression of the *poschiavinus* allele of *Sry* (our unpublished observations). Of the 14 liveborn XY^{POS} animals transgenic for *Dax:Dax*, three were phenotypically female, three were overt hermaphrodites and eight were males. All XY^{POS} animals that were not transgenic developed as males (16 out of 16). Inspection of the internal organs of the sex reversed animals showed that they possessed normal female reproductive tracts but they had unusually sized ovaries with very few primary follicles, similar to those seen in other XY females such as XY^{tdyml} mice (ref. 31). The hermaphrodites revealed different degrees of ovarian and testicular development with mixed male and female reproductive tracts. The transgenic males had normal male reproductive tracts, consistent with production of both AMH and testosterone during development. Testis weights of the four males that were measured were low (65.1 ± 2.8 mg), however, compared with their

non-transgenic XY^{POS} littermates (94.5 ± 1.9 mg), suggesting that they were derived from embryonic ovotestes.

To determine whether ovotestes were consistently being formed during development, whole-mount *in situ* hybridization, using an *Amh* probe, was performed on gonads from 14.5-d.p.c. embryos from matings between XY^{POS} males and *Dax:Dax* line 1812 females (Fig. 6). Eight out of eleven transgenic XY^{POS} embryos had ovotestes where the ovarian component ranged from a small portion at one or both poles to almost all of the gonad. Ovarian tissue was absent from the testes of the six non-transgenic XY^{POS} embryos. Similar results were obtained with *Dax:Dax* line 2182 (which expresses *Dax1* at a high level but not as much as does line 1812), where, out of a total of 16 XY^{POS} embryos, the 9 non-transgenics had normal testes but 5 of the 7 transgenic embryos had ovotestes with a significant ovarian component.

The wide variation in gonadal phenotypes seen amongst the transgenic XY^{POS} offspring could reflect both stochastic and genetic background effects. Three autosomal loci have been implicated in XY^{POS} sex reversal and mapped in a genome scan in mice segregating C57BL/6 and DBA alleles³². These loci are referred to as *tda1*, *tda2* and *tda3*. The C57BL/6 alleles favoured ovarian development and those of DBA allowed the *poschiavinus* *Sry* to induce testis development. However, even though in our crosses the transgenic mothers had a mixed genetic background derived from CBA and C57BL/10 strains, and the XY^{POS} fathers had an inbred C57BL/6 background, the CBA contribution has so far always been sufficient to ensure testis development except when the *Dax:Dax* transgene is present. Moreover, genotyping failed to reveal any correlation between sex reversal and homozygosity for *tda* alleles (data not shown). We therefore conclude that by far the strongest component giving rise to sex reversal with XY^{POS} is the presence of either the 1812 or the 2182 *Dax:Dax* transgene.

***Dax:Sry* transgene.** XX embryos carrying the *Dax:Sry* transgene also have a delay in testis cord formation compared with XY and XX *Sry* embryos (see Fig. 3). This transgene therefore appears to act in a similar way to Y^{POS} and can be considered a weak allele of *Sry*. We therefore bred *Dax:Dax* transgenic XX females with *Dax:Sry* transgenic XY males. All these experiments were carried out with the *Dax:Sry* transgenic line D:S23 in which 100% of the XX transgenics are male. When *Dax:Dax* line 1812 was used, all the F₀ XX embryos transgenic for both *Dax:Sry* and *Dax:Dax* developed as females (five out of five). These females were fertile and the two transgenes segregated independently amongst their offspring. F₁ XX offspring transgenic for only *Dax:Sry* developed as males (five out of five), showing that this transgene had not lost its activity, and those that inherited both transgenes developed as females (five out of five). Similar results have also been obtained using *Dax:Dax* line 2182, in that all three XX double-transgenic animals obtained were female.

When *Dax:Dax* line 1979 was used (these mice express about twice the level of the endogenous *Dax1* gene), one XX double transgenic developed as a female and another as a male. When *Dax:Dax* lines 102 and 1994 were used (both of which express the transgene at a lower level than the endogenous *Dax1*), however, all the XX double transgenics developed as males (Fig. 7).

Whole-mount *in situ* hybridization was used to assay for *Amh* expression in embryos from D:S23 $\times$ 1812 crosses. The developing gonads from XX double transgenic embryos showed no sign of testicular structure or *Amh* expression, clearly showing that ovary development was initiated from the beginning and that gonadal development did not pass through an ovotestis phase (Fig. 7).

This abrogation of sex reversal suggests that DAX1 competes very efficiently with SRY when expression of the latter is driven by the *Dax1* regulatory region. If the upstream factors required for *Dax1* expression are present in limiting amounts, however, this could be attributed to promoter competition, as both transgenes are expressed from identical *Dax1* regulatory sequences. We therefore crossed XY *Dax:Sry* males of line D:S23 with females of the *Dax:lacZ* line 1666, which express very high levels of *lacZ* (Fig. 2). XX double transgenic embryos developed as males (two out of two), consistent with the effect of the *Dax:Dax* transgene interfering with the downstream action of the *Dax:Sry* transgene and not with levels of expression through promoter interference.

Discussion

To determine whether *Dax1* is responsible for DSS we used a genital-ridge-specific regulatory region from the gene itself to target extra doses of *Dax1* to XY gonads. We found that high levels of exogenous *Dax1* expression retard testis differentiation in mice with a 'normal' *Sry* allele of *Mus musculus musculus* origin, but that complete sex reversal can result when weak alleles of *Sry*, either the naturally occurring *poschiavinus* allele or an *Sry* transgene, are used. This implies that *Dax1* and *Sry* act antagonistically towards each other, with increasing expression of the former leading to female development and increasing activity of the latter to male development. Thus, our data strongly support the proposal that the human homologue of *Dax1* is the gene responsible for DSS.

Several hierarchical models could have explained the sex reversal seen in patients with Xp21 duplications. It was therefore conceivable that a high level of DAX1 represses SRY transcription or that SRY could not repress more than one copy of DAX1. It now seems very unlikely, however, that either gene acts directly upstream of the other. In normal XY genital-ridge development in the mouse, both *Sry* and *Dax1* are expressed throughout the critical period before testis cord formation, and both are downregulated at the same time. Also, critically, the *Dax1* transgene led to female development when expression of *Sry* was driven by *Dax1* upstream sequences as well as

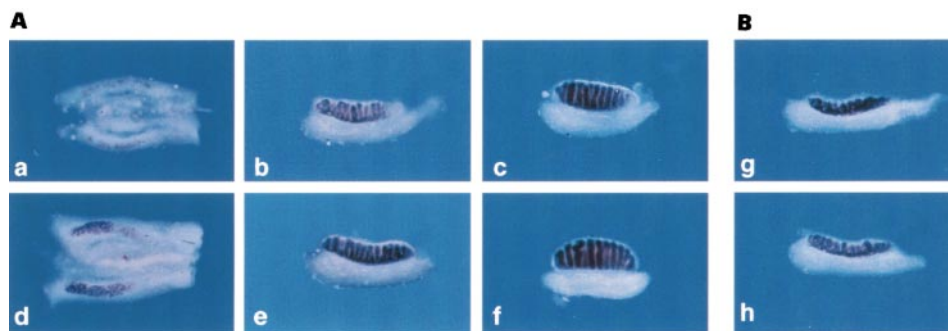


Figure 3 Delay in testis development of XX *Dax:Sry* transgenic gonads. **A**, Gonads and underlying mesonephros were dissected from 12.0 (**a**, **d**), 12.5 (**b**, **e**) and 13.5 (**c**, **f**) d.p.c. embryos and whole-mount *in situ* hybridization was performed using *Amh* as a probe. Gonads were dissected from embryos obtained from matings of XY *Dax:Sry* transgenic males and wild-type females. XX

transgenic gonads (**a**–**c**) and XY wild-type gonads (**d**–**f**) were selected for *in situ* hybridization. **B**, Gonads were derived from 12.5-d.p.c. embryos from the progeny of matings of XY males transgenic for p741.mutB (all XX transgenic offspring of this line develop as males) and wild-type females. An XX transgenic gonad (**g**) and XY wild-type gonad (**h**) were selected for *in situ* hybridization.

when *Sry* was under the control of its own regulatory sequences (in Y^{POS}). Therefore, the protein products of the two genes probably compete with each other to control target gene activation.

Although a double dose of human *DAX1* is sufficient to reverse the sex of patients with duplications of chromosome Xp21, it seems that much higher levels of expression are required for the mouse *Dax1* gene to compete effectively with *Sry* of *M. m. musculus* origin. Thus, mice homozygous for the 1812 *Dax:Dax* transgene express approximately 11 times the normal levels of *Dax1*, yet still develop as males. A formal possibility is that there is an additional gene in the 160-kb minimal DSS region that is cooperating with *DAX1* to achieve sex reversal when present in double doses. This gene would clearly be absent from the mouse *Dax1* transgene used here. But, given the results obtained with Y^{POS} and with the *Dax:Sry* transgene, there are other more likely explanations for this apparent species difference.

Sry and *Dax1* could show species differences in levels of expression relative to critical thresholds. For example, the *M. m. musculus* *Sry* gene could be expressed at significantly higher levels than the minimum required to induce testes. Indeed, the minimum level may be about 50% of normal^{4,23,33}. Perhaps the human *SRY* gene is similar to that of *poschiavinus* which appears to be expressed at a level much closer to its threshold, such that relatively small increases in *DAX1* expression are antagonistic.

Precise timing of expression of the two genes may be more critical, however. If *Dax1* expression is delayed even slightly with respect to *Sry*, it may never reach the level required to compete before *Sry* has reached its critical threshold and initiated Sertoli cell differentiation. RNase protection experiments suggested that, in XY genital ridges, the peaks of *Dax1* and *Sry* expression coincided exactly²⁰. Our *in situ* data (Fig. 1), however, show a wave of *Sry* transcription passing down the genital ridge, such that each cell expresses a high level for only a few hours, whereas *Dax1* shows more uniform expression. Most cells will therefore experience the

Sry peak before that of *Dax1*. Intriguingly, in ovotestes, ovarian differentiation often occurs at the poles and when we see a delay in *Amh* expression with high levels of *Dax1* it is the posterior pole of the genital ridge that is most affected. If *Sry* expression is delayed, high levels of *Dax1* would be able to act first at this pole.

We found that testis formation in XX *Dax:Sry* embryos is retarded in comparison with that in normal XY embryos. This is consistent with there being a delay of *Sry* expression when it is driven by the *Dax1* regulatory region. This may explain why *Dax1* competes very efficiently with *Sry* when the two genes are expressed from identical regulatory sequences. Although the time of onset of human *SRY* expression is not known, it could be relatively late. *Sry* is such a rapidly evolving gene, with little sequence conservation outside the HMG-box domain, that it is quite likely that many aspects of its regulation differ between species^{4,23}. Indeed, it is known to be expressed more widely than the mouse gene³⁴. There may also be species differences in the effective 'strength' of the gene products. *M. m. musculus* *SRY* has weak but demonstrable transactivation properties *in vitro* whereas human *SRY* does not^{35,36}. It may be relevant that the *poschiavinus* *Sry* gene (which is of the *M. m. domesticus* type) is predicted to encode a protein truncated for the carboxy-terminal putative transactivation domain^{4,37}.

Alternatively, subsequent events in testis differentiation may differ between humans and mice. In the mouse, fetal ovotestes often lose one or other component. Normally this is the ovarian tissue, such that only testicular material remains at birth (as seen with Y^{POS} mice). Perhaps in humans the bias is towards maintaining ovarian (or streak) development, so more cases of sex reversal are detected. Conversely, the data from humans could be skewed because of sampling bias and are not so different from the results in the mouse. DSS patients are identified precisely because they

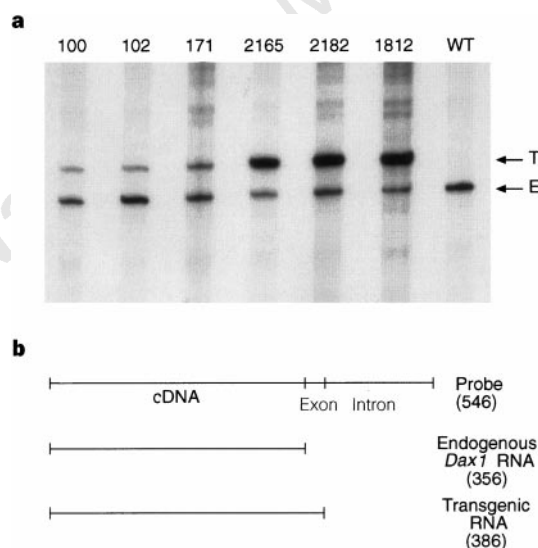


Figure 4 Expression of *Dax:Dax* transgene in the genital ridge. **a**, RNase protection analysis was done using RNA from dissected genital ridges and mesonephroi of 11.5-d.p.c. embryos resulting from matings of transgenic males and wild-type females. Each lane contains RNA from one pair of urogenital ridges from non-transgenic (wild type, WT) or transgenic embryos. The latter are named according to the number of the line of the transgenic male used. The upper band corresponds to the transgenic (T) and the lower to the endogenous (E) *Dax1* RNA. Identical results were obtained if male or female gonads were used. **b**, Diagram of the probe (top) used in the RNase protection assay and the regions of the probes that are expected to be protected from RNase digestion by the transgenic (middle) and endogenous (bottom) *Dax1* RNA.

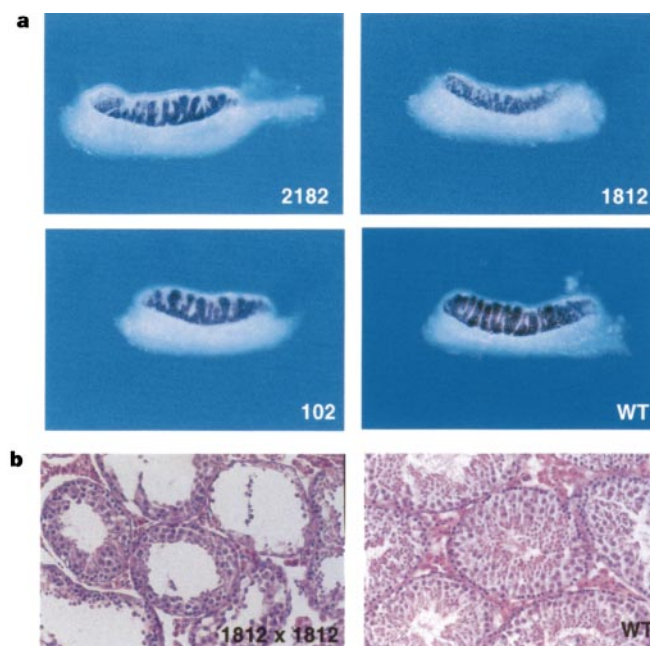


Figure 5 Effect of the *Dax:Dax* transgene on testis development. **a**, Delay in testis development of XY *Dax:Dax* transgenic gonads. Gonads and underlying mesonephros were dissected from 12.5-d.p.c. embryos and whole-mount *in situ* hybridization was performed using *Amh* as a probe. Gonads were derived from embryos from matings of transgenic males and wild-type females. XY transgenic gonads from embryos at an equal stage of development, as measured by the number of tail somites, were selected for *in situ* hybridization. Each panel shows a gonad from a numbered transgenic male line or a wild-type (non-transgenic) XY gonad (WT). **b**, Spermatogenic block in adult testis from mice homozygous for *Dax:Dax* line 1812 transgene. Sections of adult testes from transgenic and wild-type (WT) males are shown.

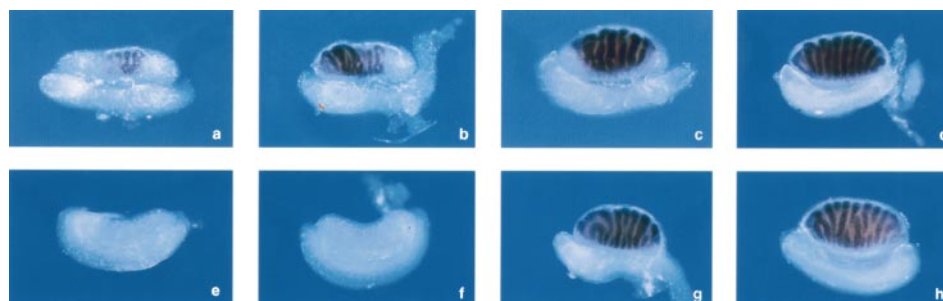


Figure 6 Effect of the *Dax:Dax* line 1812 transgene on the XY^{POS} *Sry* allele. Gonads from POS embryos transgenic for *Dax:Dax* from line 1812 show a range in the proportion of ovarian development. Gonads and underlying mesonephros were dissected from 14.5-d.p.c. embryos and whole-mount *in situ* hybridization was

performed using *Amh* as a probe. Gonads were derived from embryos resulting from matings of XY^{POS} males and *Dax:Dax* 1812 transgenic females. Gonads from non-transgenic XX and XY^{POS} embryos are shown in **e, f** and **g, h**, respectively. **a-d**, Transgenic XY^{POS} gonads.

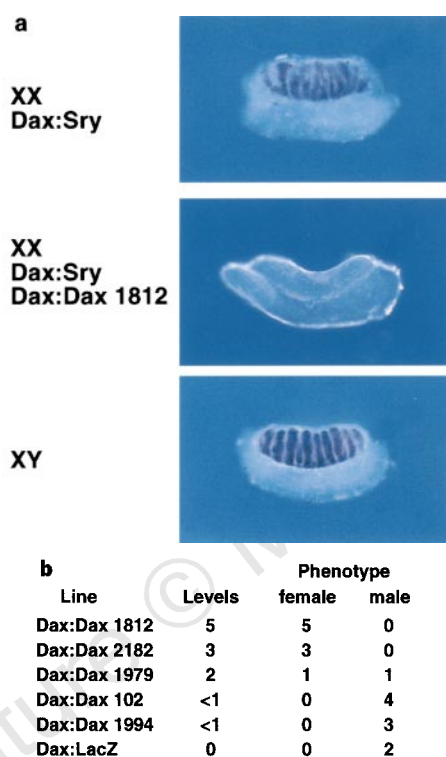


Figure 7 Effect of *Dax:Dax* transgene on *Dax:Sry*. **a**, Female development in XX embryos transgenic for *Dax:Sry* and *Dax:Dax* line 1812. Whole-mount *in situ* hybridization was performed using *Amh* to probe gonads from 13.5-d.p.c. embryos derived from matings of XY *Dax:Sry* transgenic males and XX *Dax:Dax* 1812 transgenic females. The genotypes of the gonads selected are indicated at the left of the figure. **b**, Phenotypes of XX animals transgenic for *Dax:Sry* and different alleles of *Dax:Dax*. The different alleles are indicated at the left; the levels of transgenic *Dax1* expression compared with levels or expression of endogenous *Dax1* are indicated in the column entitled 'Levels'; and the phenotype of the resulting double transgenic with the number of animals that have been analysed is indicated in the columns headed 'Phenotype'.

show sex reversal: these could be rare individuals amongst many with chromosome Xp21 duplications.

Sex-determining mechanisms are known to evolve rapidly and changes in *Sry* and *Dax1* between species may simply be part of this. It has often been proposed that X-linked genes could be involved in mammalian sex determination as vestiges of an ancient dosage-dependent sex-determining mechanism³⁸. These genes should actively promote female development; for example, pouch versus scrotum development depends on two instead of one X chromosomes in marsupials³⁹. Our data suggest, however, that *Dax1*

functions more as an 'anti-testis gene' rather than an ovary determinant. Moreover, X-chromosome inactivation means there is no *a priori* reason why *Dax1* should be on the X chromosome and, in fact, *Dax1* is autosomal in marsupials⁴⁰. It seems more likely that genes such as *Sry* and *Dax1* have imposed their control on an underlying sex-determining mechanism which may be present in all vertebrates, including those in which the switch is environmental. *Sox9* a highly conserved gene in vertebrate evolution, might be an important component of this. High expression in the gonad is associated with Sertoli cells in at least mammals and birds^{14,15}. This expression is critical because heterozygous mutations in the gene in humans frequently lead to XY female sex reversal⁴¹⁻⁴³.

How might SRY and DAX1 compete at a molecular level? Recently, evidence was obtained⁴⁴ that DAX1 can form heterodimers with a conventional member of the nuclear hormone receptor superfamily, steroidogenic factor-1 (SF-1). SF-1 is essential for early gonadal development and there is a large body of evidence implicating it in the transcriptional activation of several genes required for testis function, including steroidogenic genes and *Amh*⁴⁵⁻⁴⁷. DAX1 may act as a co-repressor, however, altering the properties of SF-1 so that it no longer activates its target genes. With the notable exception of Sertoli cells, *Dax1* and *Sf-1* are always co-expressed and the function of the former may generally be to modulate the activity of the latter⁴⁸. This allows a simple model, whereby heterodimers of DAX1 and SF-1 ensure that secondary testis determining genes, such as *Sox9*, are repressed in ovary development. In XY gonads SRY, perhaps by altering DNA conformation, prevents SF-1–DAX1 heterodimers from binding to, or assembling on, their target sequence, while SF-1 alone could still bind and transactivate. When DAX1 is present at abnormally high levels, however, or is expressed early with respect to SRY, the co-repressor complexes outcompete the binding of SF-1 and perhaps also SRY itself, such that SOX9 never reaches a critical threshold to allow Sertoli cell differentiation.

In conclusion, we have developed a transgenic model system to study events in mammalian sex determination. Our data show that *Dax1* functions as an anti-testis gene by acting antagonistically to *Sry*. The sex-determining mechanism that is being revealed is exquisitely sensitive to levels and timing of expression of both these genes. Mammalian sex determination is therefore part of a rapidly evolving system with *Dax1* and *Sry* at the top of the hierarchy.

Note added in proof: A recent paper by Zazopoulos *et al.* (*Nature* **390**, 311–315, 1997) suggests an alternative way in which DAX1 may repress SF-1-mediated transcriptional activation. However, this does not alter the essentials of the model proposed above. □

Methods

Transgenic constructs. The genomic *Dax1* DNA used in these studies was derived from a 12-kb *Bam*HI fragment comprising 11-kb mouse DNA

upstream of the start of transcription plus 750 bp of the transcribed region of *Dax1* (up to nucleotide 980 as in ref. 20). For *Dax:lacZ*, a fragment containing the *lacZ* gene and the SV40 polyadenylation signal was blunt-end-ligated into the coding region of the genomic *Dax-1* fragment, at the *MluI* site (nucleotide 304) (ref. 20), to create a fusion protein comprising 24 amino acids of *Dax1* followed by the *lacZ* protein. For *Dax:Sry*, the 5.3-kb *SspI*-*Bsu36I* (5'–3') fragment of *Sry*, which includes the coding region and the 3' UTR, was blunt-end-ligated into the *Dax1* genomic DNA at the *Bsu36I* site (nucleotide 225) (ref. 20) within the 5' UTR. For *Dax:Dax*, the rabbit β -globin intron and polyadenylation signal (*Bam*HI to *Bgl*II fragment) was inserted downstream of the coding region of the mouse *Dax1* cDNA at an *NdeI* site and this construct was then introduced into the genomic *Dax1* fragment at the *MluI* site. The p741.mutB-construct contains 13-kb genomic *Sry* DNA (*Bam*HI to *Sall* fragment) with a silent mutation within the open reading frame of *Sry* used for genotyping⁴⁹. This construct produces an identical phenotype to the 14-kb 741 *Sry* genomic fragment previously characterized in transgenic mice³.

Mouse strains and transgenic mice. The DNA fragments used for pronuclear injections were purified by gel electrophoresis and treatment with AgarACE from Promega. Eggs were obtained from CBA $\times$ C57BL/10 F₁ females mated to F₁ males and resulting transgenic lines maintained by crossing to non-transgenic F₁ animals unless otherwise stated. The C57BL/6 XY^{POS} males were from a stock originally supplied to P.S.B. by E. Eicher. Transgenic mice were identified by polymerase chain reaction (PCR) using the following primers: *Dax:lacZ*, 5' GCAGAACGAGCTACA GGAGC3' (*Dax1* 5A) and 5'-AGATGGGCGCATCGTAACCG-3' (*lacZ* pZ3); *Dax:Sry*, *Dax1* 5A and CAGCTGCTTGCTGATCTCTG (*Sry* Y33B); *Dax:Dax*, 5'-TCAAGTGTGGA GTCTGAACATTG-3' (*Dax1* 1A) and 5'-GCACAGAGCATCTCCAGCATCAT-3' (*Dax1* 3B). The embryos and newborn mice were sexed by assaying for the presence of the Y chromosome by PCR using primers specific for *Zfy-1* (ref. 4).

LacZ expression. Staining for β -galactosidase activity was carried out according to standard procedures⁵⁰. Briefly, embryos were fixed for 30 min in 2% paraformaldehyde/0.1% glutaraldehyde, washed in PBS buffer and incubated overnight at 37 °C in staining solution. Adult ovaries were treated in the same way as the embryos and were then dehydrated using ascending concentrations of ethanol, cleared in histoclear, embedded in paraffin and sectioned. Fixed adult testes were left overnight in 30% sucrose, embedded in OCT (Optimum Cutting Temperature compound) and frozen on dry ice. Cryostat sections were incubated overnight at 37 °C in staining solution, washed in PBS and mounted.

Whole-mount *in situ* hybridization. This was performed as previously described⁵¹. The *Amh*, *Dax1* and *Sox9* antisense RNA probes were used as previously described in refs 14, 20, 24. The *Sry* antisense RNA probe was generated from a DNA fragment containing 40-bp *Sry* coding region and 535-bp 3' UTR sequences (*Eco*RII to *Eco*RI fragment)⁴.

RNAse protection assay. These assays were performed as previously described⁴. The radioactively labelled antisense RNA probe was generated from a DNA fragment containing 356-bp *Dax1* cDNA (*Pvu*II to *NdeI* fragment) and 30-bp exon and 160-bp intron sequences from rabbit β -globin (*Bam*HI to *Bgl*II fragment). Quantification of the Phosphor Imager data was done using a Molecular Dynamics computer imaging system.

Histological analysis. Ovaries were fixed in Bouin's reagent for 24 h, dehydrated in ascending concentration of ethanol, cleared in xylene and embedded in paraffin wax. The embedded samples were sectioned and stained with haematoxylin and eosin.

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Correspondence and requests for materials should be addressed to R.L.-B.

Magneto-electric Aharonov-Bohm effect in metal rings

Alexander van Oudenaarden, Michel H. Devoret*, Yu. V. Nazarov & J. E. Mooij

Department of Applied Physics and DIMES, Delft University of Technology, Lorentzweg 1, 2628 CJ Delft, The Netherlands

* Permanent address: Service de Physique de l'Etat Condensé, CEA-Saclay, F-91191, Gif-sur-Yvette, France.

The quantum-mechanical phase of the wavefunction of an electron can be changed by electromagnetic potentials, as was predicted by Aharonov and Bohm¹ in 1959. Experiments on propagating electron waves in vacuum have revealed both the magnetic²⁻⁴ and electrostatic⁵ Aharonov-Bohm effect. Surprisingly, the magnetic effect was also observed in micrometre-sized metal rings⁶⁻⁸, demonstrating that electrons keep their phase coherence in such samples despite their diffusive motion. The search for the electrostatic contribution to the electron phase in

these metal rings^{9,10} was hindered by the high conductivity of metal, which makes it difficult to apply a well defined voltage difference across the ring. Here we report measurements of quantum interference of electrons in metal rings that are interrupted by two small tunnel junctions. In these systems, a well defined voltage difference between the two parts of the ring can be applied. Using these rings we simultaneously explore the influence of magnetic and electrostatic potentials on the Aharonov-Bohm quantum-interference effect, and we demonstrate that these two potentials play interchangeable roles.

To determine the combined influence of electrostatic and magnetic potentials on the quantum interference of electron waves, we consider the model shown in Fig. 1a and b. A metallic ring is interrupted by two tunnel barriers denoted by the black regions in Fig. 1b. Because the resistance of these tunnel barriers is much larger than the resistance of the metallic part, a well defined potential V can be applied across the two halves of the ring. Electric transport occurs because electrons can tunnel from an occupied state in the left half to an empty state in the right half, as shown in Fig. 1a. This process is equivalent to the creation of an electron-hole pair during a tunnel event. After tunnelling, the electron and the hole start to diffuse in the right and left halves of the ring, respectively. Because the transport is diffusive, the electron and hole have a finite probability of recombining at the other tunnel barrier as shown in Fig. 1b. Only those trajectories in which the electron and hole trajectories form a closed loop circling the ring are sensitive to the magnetic field through the ring. At the moment of recombination, the phase difference $\Delta\phi_B$ between the electron and hole due to the magnetic field B is $2\pi eBS/h$ (where e is the electron charge, h is Planck's constant, and S is the area of the ring). This results in alternating constructive and destructive interference between the electron and hole wave as a function of B , with a period $h/(eS)$. This effect has analogies with the modulation of the Cooper pair current with period $h/(2eS)$ in a superconducting ring interrupted by tunnel junctions (a superconducting quantum interference device, SQUID)^{11,12}. But in the case of the SQUID the effect results from collective Cooper pair tunnelling (the Josephson effect), whereas here the interference takes place at the single electron level. Because of the voltage difference, the electron and hole have different energies with respect to the Fermi level in both halves of our device. The sum of the energy of the hole ϵ_h and the electron ϵ_e

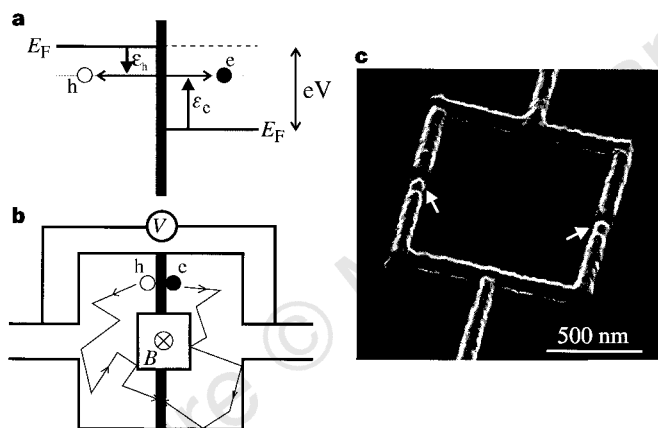


Figure 1 Quantum interference of electron-hole pair in mesoscopic ring. **a**, Energy diagram modelling a tunnelling process. At the moment of tunnelling, an electron (e) with energy ϵ_e is injected into the right part of the sample leaving a hole (h) with energy ϵ_h in the left part. **b**, Typical electron-hole trajectories which are sensitive to an Aharonov-Bohm flux. At the moment of a tunnel event an electron-hole pair is created. The hole and the electron diffuse separately to the other tunnel barrier where they recombine and interfere. The interference of the electron and hole wave is both sensitive to a magnetic field B piercing the ring (magnetic Aharonov-Bohm effect) and a voltage V across the ring (electrostatic Aharonov-Bohm effect). **c**, Scanning electron microscope image of the Aharonov-Bohm ring which is interrupted by two small tunnel junctions. The sample consists of a $0.9\ \mu\text{m} \times 0.9\ \mu\text{m}$ square loop. The width of the arms of the loop is $60\ \text{nm}$. The loop is interrupted by two small tunnel junctions of $60 \times 60\ \text{nm}^2$. The average distance L between the tunnel barriers is $1.6\ \mu\text{m}$. The mask of the loop was defined by electron beam lithography. The junctions were fabricated in three strips. First, a 25-nm -thick aluminium layer was deposited at an angle with respect to the oxidized Si substrate. Second, the aluminium was oxidized to form the insulating tunnel barriers. Finally, a second 40-nm -thick aluminium layer was deposited at another angle. The tunnel junctions are denoted by the arrows. By determining the overlap area of the different junctions, we conclude that the tunnelling conductances do not differ by more than 10%. This is confirmed by critical current measurements in the superconducting state, in which the sample operates as a SQUID. The tunnel conductance G_T of each junction is $55 \pm 5\ \mu\text{S}$, which is about four orders of magnitude smaller than the conductance of the metallic part of the ring.

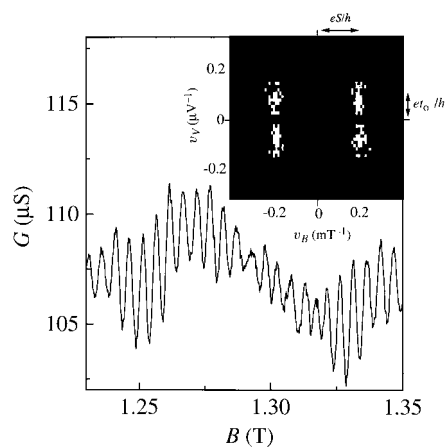


Figure 2 Conductance G versus magnetic field B at $T = 20\ \text{mK}$ and $V = 500\ \mu\text{V}$. Aharonov-Bohm oscillations with a period $B_{AB} = 5\ \text{mT}$ are observed as a function of B . This period is in agreement with the magnetic field of $5.3\ \text{mT}$ which is needed to add a flux quantum h/e to the average area $S = 0.75\ \mu\text{m}^2$ enclosed by the ring. In the inset, the Fourier power spectrum is shown as a function of the magnetic and electric 'frequencies' ν_B and ν_V . The lighter the grey scale the larger the Fourier power. The magnetic and electric field modulation of G_{AB} manifests itself as peaks around $\pm 0.2\ \text{mT}^{-1}$ and $\pm 0.1\ \mu\text{V}^{-1}$, respectively.

equals eV (Fig. 1a). Owing to this energy difference, the electron–hole pair accumulates an electrostatic phase difference $\Delta\phi_V = 2\pi eVt/h$, where t is the time the electron and hole spend in their respective parts of the ring before they recombine. Because electron motion is diffusive there is not one unique time, but rather a distribution of times with an average value $t_0 = L^2/D$ where L is the distance along the ring between the two tunnel barriers and D is the diffusion coefficient. The electrostatic interference results in an alternating constructive and destructive interference as a function of V with a period $h/(et_0)$. Experimentally, one can explore both the magnetic and electrostatic quantum interference effect by measuring the Aharonov–Bohm flux-dependent part G_{AB} of the conductance. The magnetic field B and the bias voltage V have an equivalent effect on G_{AB} . Changing the magnetic field at fixed V leads to a periodically oscillating G_{AB} with a period which is solely defined by the geometry of the ring. Measuring the conductance as a function of V at fixed B results in a periodically oscillating G_{AB} with an average period which is determined by t_0 . In other words, by exploring the voltage dependence of G_{AB} , t_0 is measured experimentally. In the ballistic regime related interference experiments¹³ have been performed in which one of the arms of the Aharonov–Bohm ring was interrupted by a quantum dot. But the relevant interference processes in such systems, where the number of electrons in the dot is changing, differ significantly from the interference in the ring we consider here.

To investigate the combined role of magnetic and electrostatic potentials we designed the sample shown in Fig. 1c. The differential conductance G at a voltage V and magnetic field B was measured using a lock-in technique. Details of the measurement set-up are given in ref. 14. All measurements were performed at $B > 1$ T to drive the aluminium loop into the normal state. At those fields time-reversal symmetry is broken, and effects related to this symmetry can be neglected. In Fig. 2 the conductance G is plotted as a function of B at a bias voltage $V = 500 \mu\text{V}$ and a temperature $T = 20$ mK. Clear periodic oscillations of the conductance are observed with a period of 5 mT, which is in good agreement with the predicted period for h/e oscillations. The relative amplitude of the Aharonov–Bohm oscillations at $V = 500 \mu\text{V}$ is $\sim 5\%$, which is considerably larger than the results obtained for uniform rings^{6–10}. The magnetic field not only pierces the hole of the loop, but also penetrates the

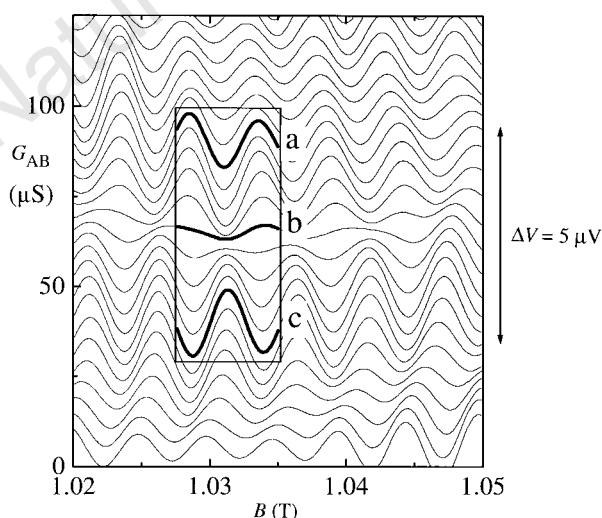


Figure 3 Aharonov–Bohm conductance as a function of magnetic field B at $T = 20$ mK for bias voltages separated by $0.48 \mu\text{V}$. The traces have been offset by $5 \mu\text{S}$ with respect to the conductance-axis for clarity. The Aharonov–Bohm conductance G_{AB} has been extracted from G by rejecting the Fourier components of G for magnetic frequencies smaller than 0.1 mT^{-1} . The bold traces a–c in the rectangular box show an example of the combined manifestation of the magnetic and electrostatic Aharonov–Bohm effect.

arms of the loop in contrast to the ideal geometry proposed by Aharonov and Bohm¹. This gives rise to aperiodic conductance fluctuations¹⁴, clearly visible in Fig. 2 as the slowly varying background on top of the Aharonov–Bohm oscillations. Because of the large difference in magnetic field scales, it is possible to filter out the aperiodic conductance fluctuations using Fourier analysis. In this way we can extract (from the conductance G) the Aharonov–Bohm conductance G_{AB} , which we would measure if the field was applied only inside the ring. The Fourier power spectrum of G_{AB} with respect to B and V is shown in Fig. 2 inset.

Figure 3 shows how the Aharonov–Bohm conductance G_{AB} evolves when the bias voltage is changed. The voltage increment between two successive traces is $0.48 \mu\text{V}$. Trace a in Fig. 3 denotes a minimum of the Aharonov–Bohm conductance at $V = 519 \mu\text{V}$. When the voltage is decreased by $2.5 \mu\text{V}$ (trace b) the Aharonov–Bohm oscillations have almost vanished. A further decrease of the voltage by $2.5 \mu\text{V}$ (trace c) leads again to an increase of the oscillations. However, the minimum near $B = 1.032$ T for $V = 519 \mu\text{V}$ (trace a) turned into a maximum at $V = 514 \mu\text{V}$ (trace c). This observation unambiguously demonstrates the symmetry between the magnetic and the electrostatic Aharonov–Bohm effect. A maximum G_{AB} (constructive electron–hole interference) is turned into a minimum G_{AB} (destructive electron–hole interference) either by increasing the magnetic field by 2.5 mT or by increasing the voltage by $5 \mu\text{V}$. This symmetry is also reflected in the Fourier power spectrum (Fig. 2 inset) by the peaks around $\pm 0.2 \text{ mT}^{-1}$ and $\pm 0.1 \mu\text{V}^{-1}$.

To obtain a more quantitative analysis, we calculated the correlation function $C(\Delta B, \Delta V) = \langle G_{AB}(B, V)G_{AB}(B + \Delta B, V + \Delta V) \rangle$, where the angle brackets denote an ensemble average. The quantity is shown in Fig. 4. The squares in Fig. 4 denote the experimental $C(\Delta B, \Delta V = 0)$. Its oscillatory behaviour is again the manifestation of the magnetic Aharonov–Bohm effect (we denote the period by B_{AB}). The triangles in Fig. 4 denote the experimental cross-correlation

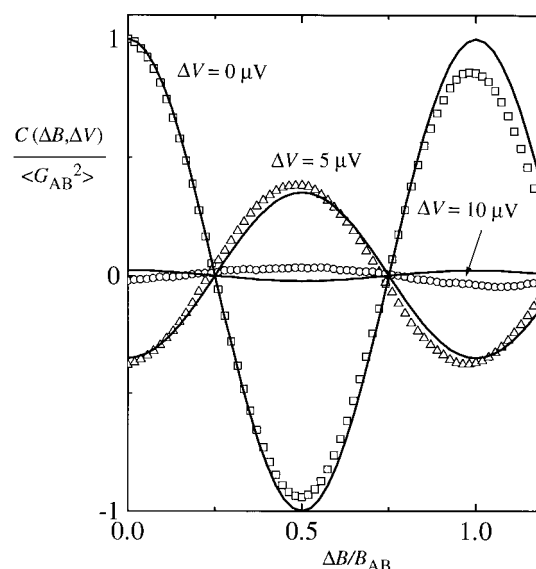


Figure 4 Normalized correlation function versus $\Delta B/B_{AB}$ at $T = 20$ mK. The correlation function $C(\Delta B, \Delta V) = \langle G_{AB}(B, V)G_{AB}(B + \Delta B, V + \Delta V) \rangle$ has been normalized to the variance $C(0, 0) = \langle G_{AB}^2 \rangle$. Squares, triangles and circles correspond to $\Delta V = 0, 5$ and $10 \mu\text{V}$, respectively. The correlation functions are calculated from a total data set consisting of 350 conductance traces as a function of B between 1.0 and 1.1 T or 2.0 and 2.1 T. These traces are measured at different voltages in the range 395–533 μV . The theoretical correlation function depends only on two parameters: t_0 and τ_ϕ , which is the time during which an electron–hole pair preserves its phase memory. A close agreement between the experimental results and theory (full lines) is found for $t_0 = 300$ ps and $\tau_\phi = 300$ ps. These values are consistent with previous experiments¹⁴.

function between two traces which are measured at bias voltages which differ by $5\ \mu\text{V}$ (for example, trace a and c in Fig. 3). The correlation is maximum at $\Delta B = \frac{1}{2}B_{AB}$; this means that by increasing the bias voltage by $5\ \mu\text{V}$ a minimum G_{AB} is turned into a maximum G_{AB} . At $\Delta V = 10\ \mu\text{V}$ (circles) the correlation is almost completely lost. The electron–hole pair cannot return to its creation point coherently. This is consistent with the absence of the $h/2e$ oscillations as a function of magnetic field (data not shown). When we compare our experimental results directly to the theory (shown as solid lines in Fig. 4) given in refs 14 and 15, close agreement is found.

These measurements demonstrate that the quantum-mechanical phase of diffusive electrons in metals is affected not only by a magnetic field, but also by an electrostatic field as predicted by Aharonov and Bohm. Whereas the period of the magnetic Aharonov–Bohm effect is given by the ratio of the magnetic flux through the ring to the flux quantum h/e , the period of the electrostatic Aharonov–Bohm effect is given by the ratio of the electrostatic ‘Thouless flux’ VL^2/D to h/e . This type of experiment enables one to directly measure the average diffusion and phase breaking time of electrons in metals. □

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Correspondence and requests for materials should be addressed to A.v.O. (e-mail: socrates@qt.tn.tudelft.nl).

Noise-supported travelling waves in sub-excitable media

Sándor Kádár, Jichang Wang & Kenneth Showalter

Department of Chemistry, West Virginia University, Morgantown, West Virginia 26506-6045, USA

The detection of weak signals of nonlinear dynamical systems in noisy environments may improve with increasing noise, reaching an optimal level before the signal is overwhelmed by the noise. This phenomenon, known as stochastic resonance^{1,2}, has been characterized in electronic³, laser⁴, magnetoelastic⁵, physical⁶ and chemical⁷ systems. Studies of stochastic resonance and noise effects in biological^{8,9} and excitable dynamical systems^{10–13} have

attracted particular interest, because of the possibility of noise-supported signal transmission in neuronal tissue and other excitable biological media. Here we report the positive influence of noise on wave propagation in a photosensitive Belousov–Zhabotinsky^{14–17} reaction. The chemical medium, which is sub-excitable and unable to support sustained wave propagation, is illuminated with light that is spatially partitioned into an array of cells in which the intensity is randomly varied. Wave propagation is enhanced with increasing noise amplitude, and sustained propagation is achieved at an optimal level. Above this level, only fragmented waves are observed.

Experiments were carried out with thin layers of silica gel in which ruthenium(II)-bipyridyl, a light-sensitive catalyst for the Belousov–Zhabotinsky (BZ) reaction, was immobilized¹⁸. The gel was continually bathed with fresh, catalyst-free BZ reagents in a Plexiglas reactor to maintain the system in an unvarying non-equilibrium state¹⁹. Gels were exposed (from below) to spatially homogeneous light transmitted from a video projector through a 460-nm bandpass filter. The light intensity was adjusted to an appropriate reference level for maintaining the system slightly below the excitability threshold (determined by the photochemical production of Br^- ion^{16,20}, an inhibitor of autocatalysis in the BZ reaction). The illumination field consisted of an array of square cells, with the intensity in each adjusted at equal time intervals to random values above or below the reference level. The random intensities were gaussian-distributed with the mean amplitude centred at the reference intensity.

The effects of the spatiotemporal noise on wave propagation are shown in Fig. 1, where superimposed ‘snapshots’ depict the evolution of a single wave segment at equal time intervals. The noise level was varied from zero in Fig. 1a, corresponding to the noise-free autonomous system, to the maximum level in Fig. 1d, which was determined by the reference intensity. A typical noise pattern is shown in Fig. 1e, where the boundary was provided by light intensity at the reference level. Waves were generated in the adjacent region, with a lower light intensity (and higher excitability), at the left-hand side. In the absence of noise, waves entering the sub-excitable region became shrinking wave segments (Fig. 1a). Below the excitability threshold, free wave ends simply recede rather than form the familiar rotating spiral waves observed in excitable media^{21,22}. Propagation failure occurs when the receding wave disappears, as shown in the final frame of the overlaid images. A slight enhancement of wave propagation is observed with low-level noise (Fig. 1b), in which wave failure occurs about one frame later. At higher noise levels (Fig. 1c), the wave segment no longer shrinks but propagates through the entire medium. At still higher levels (Fig. 1d), fragmentation of the wave occurs at wave breaks induced by high-intensity perturbations.

The dependence of the signal strength on noise level and cell size is summarized in Fig. 2. The relative signal strength was defined by the size of the wave segment (number of pixels above a specified grey level) at the point of wave failure in the autonomous system relative to the size of the wave segment entering the sub-excitable region. An increase in the noise level results in an increase in the size of the wave segment passing through the detection point, reaching a maximum at an optimum noise level. Wave propagation also depends on the degree of spatial correlation of the noise, which is determined by the cell size. For a cell size (4×4 pixels) comparable to the length scale of the wave width, the optimal noise level is ~ 0.6 of the maximum level. The optimal noise level increases as the cell size decreases, to ~ 0.8 of the maximum level for cells of 2×2 pixels. No optimal level was found, within our experimental noise range, when the cell size was defined by one pixel. Related trends in spatial correlation dependence have been found in theoretical studies of stochastic resonance in excitable media^{12,13}.

Numerical studies of noise-supported wave propagation were carried out using the Tyson–Fife²³ scaling of the Oregonator

model²⁴, modified to describe the photosensitive BZ reaction^{25,26}:

$$\begin{aligned}\frac{\partial u}{\partial t} &= \delta \nabla^2 u + (1/\epsilon)(qw - uw + u - u^2) \\ \frac{\partial v}{\partial t} &= u - v \\ \frac{\partial w}{\partial t} &= \nabla^2 w + (1/\epsilon')(\phi - qw - uw + fv)\end{aligned}\quad (1)$$

Here u , v and w are the dimensionless concentrations of HBrO_2 , $\text{Ru}(\text{bpy})_3^{3+}$ and Br^- , respectively; ∇^2 is the laplacian operator; $\delta = D_u/D_w$ is the ratio of the diffusion coefficients for u and w ; ϵ , ϵ' and q are scaling parameters; f is an adjustable stoichiometry parameter; and ϕ represents the rate of bromide production from the irradiation. This rate is proportional to the applied light intensity²⁰ and is used as the control parameter determining the excitability. As in the experiments, the excitability of each cell in an array of cells is adjusted at equal time intervals by varying the value of ϕ to random levels above or below the reference level.

The effects of increasing noise level on the simulated wave propagation are shown in Fig. 3, where, again, the evolution is depicted by superimposed snapshots. The parameters were chosen such that the system does not support sustained wave propagation in the absence of noise, and waves entering the sub-excitable medium recede and disappear (Fig. 3a). When low-level noise is applied, the wave segment travels beyond the failure point in the autonomous system but fails a few image frames later (Fig. 3b). As observed in the experiments, there is an optimal, higher noise level, where sustained wave propagation appears, as shown in Fig. 3c. At

still higher noise levels, the wave segment is subjected to strong inhibition and stimulation at the local cell level, giving rise to perturbation-induced wave breaks (Fig. 3d). The behaviour is highly variable, but, typically, only small wave segments survive and often there is complete annihilation of wave activity. Calculations carried out to determine the dependence on cell size showed a maximum in relative signal strength (as defined above) at a cell size comparable to the wave width. Calculations were also carried out to determine the dependence on the noise timescale. For a particular cell size (10×10 pixels) and noise level (0.6), the relative signal strength had a maximum at a noise refresh time corresponding to ~ 2 cycles as the wave passed through a single cell or ~ 75 cycles before the wave reached the failure point of the autonomous system. Support for wave propagation falls off at increasing noise frequencies because the slower reaction–diffusion system cannot respond to each perturbation but rather experiences an average of the perturbations, which is the sub-excitable state. Support also falls off at decreasing noise frequencies because the wave must then percolate through noise patterns that approach a static state.

Our experiments and calculations show that noise supports wave propagation in sub-excitable media by affecting the excitability threshold²⁷. A phase-plane analysis of equations (1), with diffusion neglected, shows that perturbations of the intensity above or below the reference intensity give rise to shifts that decrease or increase the excitability threshold. Near the optimal noise level, perturbations increasing the excitability tend to be amplified, as the system responds to sufficiently large perturbations with large-amplitude oscillations. Perturbations decreasing the excitability have a smaller effect, as such perturbations from the photo-stationary state simply

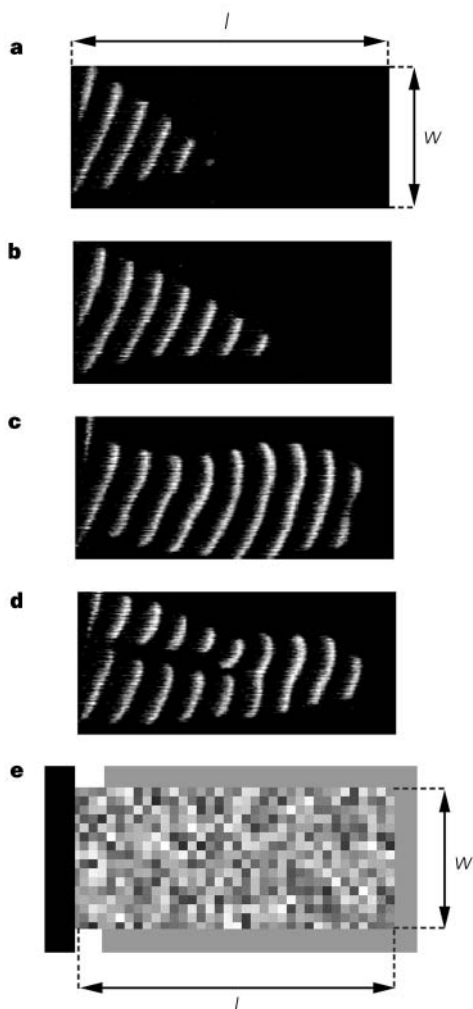


Figure 1 Image sequences showing influence of noise on wave propagation. Images of wave segments travelling through BZ medium perturbed by 0 (**a**), 0.3 (**b**), 0.6 (**c**) and 1.0 (**d**) of maximum noise level permitted by experimental conditions, and an example of the imposed noise array (**e**), with an adjacent excitable region (black) for initiating waves and the surrounding boundary (grey) at the sub-excitable reference intensity. The superimposed snapshots show behaviour at 20.1-s intervals; the noise array was updated at 6.7-s intervals. The area of imposed noise ($l = 12$ mm; $w = 5.2$ mm) in the illumination field consisted of an array of square cells with the intensity I in each adjusted at equal time intervals to random values according to $I = I_0(1 + \frac{1}{2}G(x, \sigma))$ with $|x| \leq 2\sigma$, where I_0 is the reference intensity and $G(x, \sigma)$ is a gaussian distribution with a standard deviation of σ . The noise level, given by the value of σ , was varied between 0 and 1.0, and for a particular noise level, the intensity I was varied on an eight-bit grey scale between 0 and 255. The cell size in these images was 4×4 pixels (0.33×0.33 mm). The medium was illuminated with light from a video projector passing through a 460-nm bandpass filter and was monitored with a CCD video camera. Images were collected during the 50-ms interval between successive updatings of the imposed noise pattern. Composition of BZ solution: 0.27 M NaBrO_3 , 0.05 M malonic acid, 0.15 M bromomalonic acid and 0.15 M H_2SO_4 . The silica gel medium ($0.3 \times 20 \times 30$ mm) was prepared by acidifying a solution of 10% (w/w) Na_2SiO_3 and 0.5 mM $\text{Ru}(\text{bpy})_3^{2+}$ with H_2SO_4 . Images of successive wave behaviour are available at <http://heracles.chem.wvu.edu/noisewaves>.

decay and during a large-amplitude oscillation they have no significant effect. As the noise level is further increased, however, these perturbations become sufficiently strong to drive the excited system back to the steady state, effectively quenching a large-amplitude oscillation. Qualitatively similar features can also be discerned in the reaction–diffusion system by monitoring phase-plane trajectories at a particular point in space as a wave passes through the medium. A large-amplitude oscillation corresponds to

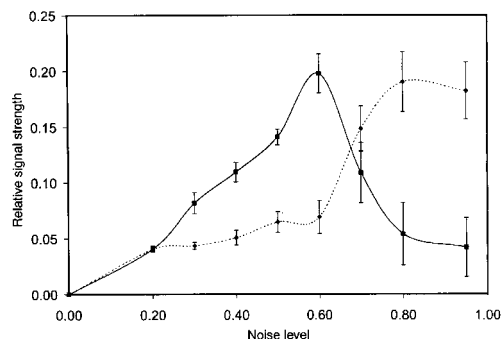


Figure 2 Dependence of relative signal strength on noise level. The solid curve shows behaviour with a noise array made up of 4×4 pixel cells (0.33×0.33 mm); the dotted curve shows behaviour with 2×2 pixel cells (0.16×0.16 mm). Points show mean values from 12–15 measurements and bars indicate standard deviations.

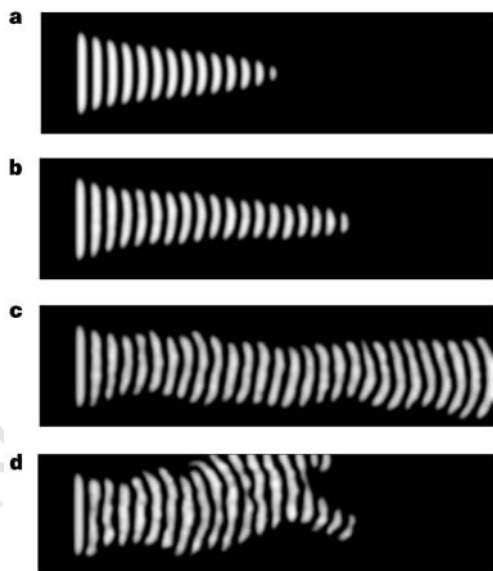


Figure 3 Images showing evolution of wave segments. The wave behaviour was calculated from equations (1) with noise levels 0 (a), 0.3 (b), 0.6 (c) and 1.0 (d). Concentrations of u are shown by proportional grey levels, with white assigned to the highest value and black assigned to the steady-state value. The medium consisted of an array of square cells with the value of ϕ in each adjusted at equal time intervals to random values according to $\phi = \phi_0(1 + \frac{1}{2}G(x, \sigma))$ with $|x| \leq 2\sigma$, where ϕ_0 is the reference value and $G(x, \sigma)$ a gaussian distribution with a standard deviation of σ . The noise level is given by the value of σ . The cell size was 10×10 pixels (0.8×0.8 dimensionless space units). The time interval between superimposed images is 0.704 and the noise array was updated at intervals of 0.128 (dimensionless time units). Equations (1) were integrated in a two-dimensional rectangular grid ($N_x \times N_y = 750 \times 200$) by an explicit Euler method (unit grid size $\Delta h = 0.08$, time step $\Delta t = 6.4 \times 10^{-5}$) with no-flux boundary conditions and a five-point approximation of the laplacian. Parameters: $\epsilon = 0.03$, $\epsilon' = 0.0003$, $q = 0.0015$, $f = 1.0$, $\phi = 0.05985$, $D_u = D_w = 1.0$, $D_v = 0$. The behaviour remained unchanged when the time step was reduced by a factor of two or when the boundary conditions were changed to mimic the experiment with the surrounding region illuminated at the reference level intensity.

a passing wave, and a perturbation suppressed oscillation corresponds to a wave break.

The quantitative measurements of relative signal strength as a function of noise level, shown in Fig. 2, display trends much like those observed in stochastic resonance experiments^{8,9}: there is an optimal noise level providing maximum support for wave propagation, and coherent wave propagation tends to be overwhelmed at high noise levels. Successive waves periodically entering the sub-excitable region from a spiral wave source in the adjacent excitable medium provide an even closer link to the amplification of a weak periodic signal by noise. There are important differences, however, between classical stochastic resonance and noise-supported waves in sub-excitable media: a qualitative change in behaviour occurs near the optimal noise level, where the medium develops the capability of supporting sustained wave propagation. Another qualitative change in behaviour occurs at higher noise levels with the advent of perturbation-induced wave breaks.

Signal transmission by excitable media in biological systems is known to be affected by noise^{8,9}. The response of sub-excitable chemical media suggests that noise might play a vital role in wave propagation in biological media with similar dynamical characteristics, such as calcium waves in networks of glial cells. Indeed, noise-supported waves in astrocyte syncytia have been observed, suggesting a mechanism for long-range signalling in neural networks²⁸. □

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Correspondence and requests for materials should be addressed to K.S. (e-mail: kshowalt@wvu.edu).

High-strength alkali-resistant sintered SiC fibre stable to 2,200 °C

Toshihiro Ishikawa, Yasuhiko Kohtoku, Kiyoshi Kumagawa, Takemi Yamamura & Toshio Nagasawa

Ube Research Laboratory, Corporate Research & Development, Ube Industries Ltd, 1978-5 Kogushi, Ube City, Yamaguchi 755, Japan

The high-temperature stability of SiC-based ceramics has led to their use in high-temperature structural materials and composites^{1–3}. In particular, silicon carbide fibres are used in tough fibre-reinforced composites. Here we describe a type of silicon carbide fibre obtained by sintering an amorphous Si–Al–C–O fibre precursor at 1,800 °C. The fibres, which have a very small aluminium content, have a high tensile strength and modulus, and show no degradation in strength or change in composition on heating to 1,900 °C in an inert atmosphere and 1,000 °C in air—a performance markedly superior to that of existing commercial SiC-based fibres such as Hi-Nicalon. Moreover, our fibres show better high-temperature creep resistance than commercial counterparts. We also find that the mechanical properties of the fibres are retained on heating in air after exposure to a salt solution, whereas both a representative commercial SiC fibre and a SiC-based fibre containing a small amount of boron were severely degraded under these conditions⁴. This suggests that our material is well suited to use in environments exposed to salts—for example, in structures in a marine setting or in the presence of combustion gases containing alkali elements.

Si–Al–C–O fibre was synthesized using polyaluminocarbosilane prepared by the reaction of polycarbosilane ($-\text{SiH}(\text{CH}_3)-\text{CH}_2-$) with aluminium(III)acetylacetonate. The reaction of the last two compounds proceeded at 300 °C in a nitrogen atmosphere by the condensation reaction of Si–H bonds in polycarbosilane and the ligands of aluminium(III) acetylacetonate accompanied by the evolution of acetylacetone⁵; the molecular weight then increased

by the cross-linking reaction with the formation of a Si–Al–Si bond.

Polyaluminocarbosilane was melt-spun at 220 °C, and then the spun fibre was cured in air at 160 °C. The cured fibre was continuously fired in inert gas up to 1,300 °C to obtain an amorphous Si–Al–C–O fibre. This fibre contained a non-stoichiometric excess of carbon and oxygen of ~12 wt%. The Si–Al–C–O fibre was converted into a sintered SiC fibre by decomposition accompanied by the release of CO gas at temperatures from 1,500 to 1,700 °C and sintering at temperatures over 1,800 °C. In this sintering process, aluminium plays a very important role as a sintering aid. However, to obtain a very strong sintered SiC fibre, the content of aluminium in the fibre has to be controlled under 1 wt%. As can be seen from Fig. 1, such a sintered SiC fibre (with <1 wt% Al) showed a smooth surface (Fig. 1a) and a densified structure. Moreover, in this case, the sintered SiC fibre showed transcrystalline fracture behaviour (Fig. 1b). On the other hand, a sintered fibre with a large amount of aluminium showed intercrystalline fracture behaviour (Fig. 1d). These phenomena are presumed to be related to the upper concentration limit of solid-soluble aluminium in the SiC crystal⁶. From the transmission electron microscopy (TEM) image (Fig. 1c) of the low-Al sintered SiC fibre, no obvious second phase is observed at the grain boundary and triple point. EDX (energy-dispersive X-ray) spectra taken at these places did not indicate the presence of aluminium within the detectability limit (~0.5 wt%) for the EDX system used.

The sintered SiC fibre showed high tensile strength and modulus of >2.5 GPa and >300 GPa, respectively. This mechanical strength was maintained up to 1,900 °C, and 80% of the initial strength was preserved after heat-treatment at 2,000 °C for 1 hour in Ar. On the other hand, the strength of a representative commercial SiC fibre (Hi-Nicalon) was reduced to 65% and 40% of the initial strength by heating in Ar for 1 hour at 1,550 °C and 1,800 °C, respectively. Furthermore, the relative strength of our sintered SiC fibre exposed in air at 1,000 °C and 1,300 °C for 100 hours was 100% and 55%, respectively, whereas that of Hi-Nicalon exposed under the same testing condition was 73% or 23%, respectively. From these results, this fibre is found to show excellent heat resistance and oxidation resistance compared to all types of SiC-based continuous fibre. This thermal stability of the sintered SiC fibre is assumed to be caused by the densified and sintered structure composed of nearly stoichiometric SiC. Moreover, as can be seen from Fig. 2, the sintered SiC fibre showed very little weight loss up to 2,200 °C in inert gas. Its conspicuous stability up to a very high temperature is to be

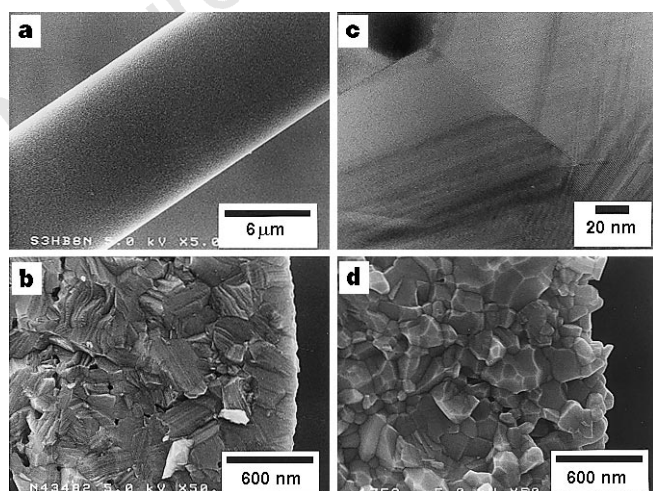


Figure 1 SEM micrographs and TEM image of our sintered SiC fibre. **a** and **b**, SEM micrographs of the surface (**a**) and cross-section (**b**; transcrystalline fracture behaviour) of our sintered SiC fibre including 0.6 wt% Al. The TEM image of our fibre is shown in **c**. To conduct a comparative study, a SEM micrograph of a sintered SiC fibre including a relatively large amount of aluminium (1.2 wt%) is shown in **d** (intercrystalline fracture behaviour). SEM micrographs and the TEM image were obtained with a Hitachi S-5000 operating at 20 kV, and a JEOL 4000EX operating at 3,000 kV, respectively.

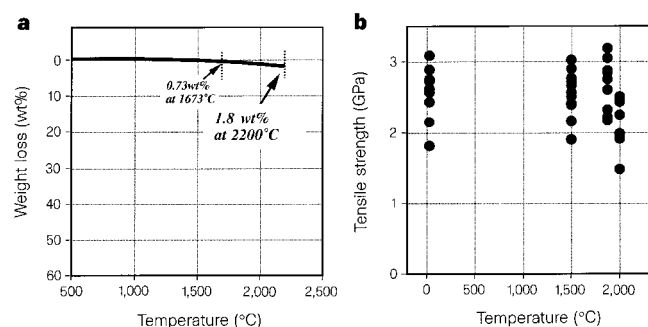


Figure 2 High-temperature performance of our sintered SiC fibre. **a**, Weight change up to 2,200 °C in He. This analysis was conducted using a TG 92 thermoanalyser; the fibre was under a helium flow, and heated from room temperature up to 2,200 °C at 10 °C min⁻¹. A graphite crucible was used as the sample vessel. **b**, Tensile strength after heat-treatment at designated temperatures up to 2,000 °C for 1 h in Ar. The tensile strengths were measured by a monofilament method using a universal testing machine (Orientec Corporation) Tension UTM-II-20 (25 mm gauge length and 2 mm min⁻¹ cross-head speed at room temperature). The diameters of the fibre were determined using a microscope with a Nikon dynascope.

expected. The very small amount of weight loss (~ 1.8 wt%) up to $2,200^\circ\text{C}$ is presumed to be related to the residual oxygen (~ 0.5 wt%), which is assumed to be captured by Si and/or Al, in the sintered SiC fibre. In this case, it seems that release of a very small amount of CO and/or SiO occurs as follows.

- (1) $\text{SiO}_2 + 3\text{C} \rightarrow \text{SiC} + 2\text{CO}$ ($\Delta G^\circ < 0$ at temperatures $> 1,538^\circ\text{C}$)
- (2) $2\text{Al}_2\text{O}_3 + 9\text{C} \rightarrow \text{Al}_4\text{C}_3 + 6\text{CO}$ ($\Delta G^\circ < 0$ at temperatures $> 1,998^\circ\text{C}$)
- (3) Vaporization of SiO (at temperatures $> 1,300^\circ\text{C}$)

This type of SiC-based fibre has been developed mainly for high-temperature applications. Therefore, its heat resistance, creep resistance and chemical resistance under severe conditions at high temperatures are very important. Figure 3 shows the creep resistance of the sintered SiC fibre in comparison with other SiC-based fibres. To obtain this result, the bending-stress relaxation testing method recommended by DiCarlo⁷ was used. In this method, the

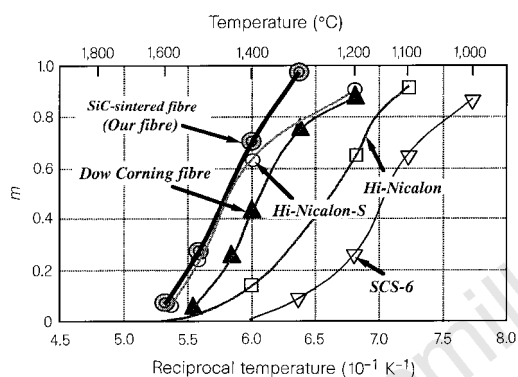


Figure 3 One-hour stress relaxation ratio of the sintered SiC fibre and other well-known SiC-based fibres. See text for details and definition of m . Data for other fibres were taken from Takeda and Imai¹³ and DiCarlo⁷. In this testing, the carbon rod ($R_0 = 8$ mm) with the wound fibre was put on a carbon surface with the same curvature surface as the rod, and then heated at each temperature in Ar for 1 h.

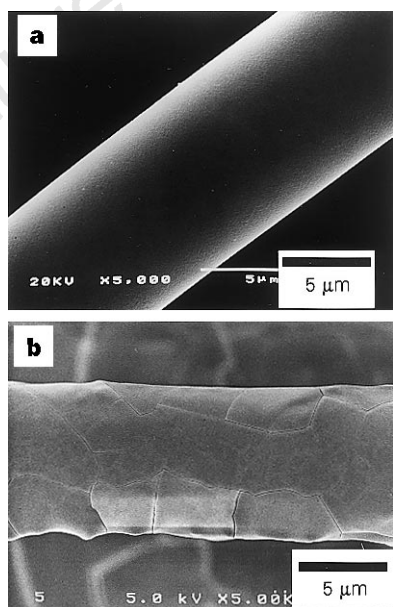


Figure 4 SEM micrograph of the surface of each fibre which had been immersed in deionized water saturated with NaCl at room temperature, and then annealed at $1,000^\circ\text{C}$ in air for 2 h. **a**, Desirable sintered SiC fibre including 0.6 wt% aluminium; **b**, SiC crystalline fibre including 1.2 wt% B, which was synthesized to conduct a comparative study.

fibre is wound around a graphite rod with a radius of R_0 and heat-treated in Ar for 1 hour. Then the stress relaxation ratio, m , is calculated using the radius, R , of the relaxed fibre from the following equation:

$$m = 1 - (R_0/R)$$

The value of m is therefore between 0 and 1. The closer to 1 the value of m , the better the creep resistance. As can be seen from Fig. 3, the sintered SiC fibre showed a higher value of m than any other SiC-based fibre. Moreover, in the case of the sintered SiC fibre, very little creep was observed even at $1,300^\circ\text{C}$. We note that both Hi-Nicalon-S⁸ and Dow Corning fibre⁹ (Fig. 3) are well-known SiC-based fibres with very high heat resistance: however, our sintered SiC fibre is found to show better creep resistance than these commercial fibres.

We have studied the alkali resistance of the sintered SiC fibre. SiC ceramics are well-known as very stable materials at high temperatures in air. The good oxidation resistance of SiC in air is due to the protection provided by the layer of vitreous silica (oxidation product). However, in the case of ordinary SiC materials, the silica can devitrify rapidly in the presence of alkali elements, which resulted in enhanced oxidation. When these SiC materials are used near the ocean or in a combustion gas containing alkali elements, these phenomena cause serious problems. Therefore, we examined the stability of our sintered SiC fibre in the presence of a salt of an alkali metal. To study the effect of salt on the fibre at high temperatures in air, the following experiments were conducted⁴. The sintered SiC fibre was immersed for 15 min in deionized water saturated with NaCl at room temperature, and then annealed at $1,000^\circ\text{C}$ for 2 hours in air. At the same time, in order to conduct a comparative study, a SiC crystalline fibre including 1.5 wt% of boron, prepared by heat-treating an amorphous Si-C-O fibre under an argon gas atmosphere including B_2O_3 vapour, was also tested in the same way. Figure 4 shows micrographs of the surfaces of the tested fibres, obtained using field-emission scanning electron microscopy (FE-SEM). As can be seen from this micrograph, the sintered SiC fibre including a small amount of aluminium has a very smooth surface. On the other hand, the SiC crystalline fibre including 1.5 wt% boron was extensively oxidized, and many cracks can be observed on the surface. Both aluminium and boron with carbon are well-known as good sintering aids for SiC crystal^{10,11}. But from the result of the above test, aluminium is found to be suitable for the synthesis of alkali-resistant, sintered SiC fibres. The difference in oxidation behaviour between the two fibres is presumed to be related to the basicity of the oxide material formed by oxidation of our aluminium-containing fibre: Al_2O_3 is an amphoteric material. Generally, materials with amphoteric or basic oxides show good alkali resistance compared to materials with acidic oxides¹². In our comparative study, the strength of a representative SiC fibre (Hi-Nicalon), which does not include either boron or aluminium, was enormously reduced—to below 10% of the initial strength—under the above testing conditions. The inclusion of a small amount of aluminium in the sintered SiC fibre therefore seems to play a very important role in obtaining very good oxidation resistance in the presence of an alkali element. □

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Correspondence and requests for materials should be addressed to T.I. (e-mail: 24613u@ube-ind.co.jp)

DNA-templated assembly and electrode attachment of a conducting silver wire

Erez Braun*, Yoav Eichen†‡, Uri Sivan*‡ & Gdalyahu Ben-Yoseph*‡

* Department of Physics, † Department of Chemistry, ‡ Solid State Institute, Technion-Israel Institute of Technology, Haifa 32000, Israel

Recent research in the field of nanometre-scale electronics has focused on two fundamental issues: the operating principles of small-scale devices, and schemes that lead to their realization and eventual integration into useful circuits. Experimental studies on molecular¹ to submicrometre² quantum dots and on the electrical transport in carbon nanotubes^{3–5} have confirmed theoretical predictions^{6–8} of an increasing role for charging effects as the device size diminishes. Nevertheless, the construction of nanometre-scale circuits from such devices remains problematic, largely owing to the difficulties of achieving inter-element wiring and electrical interfacing to macroscopic electrodes. The use of molecular recognition processes and the self-assembly of molecules into supramolecular structures^{9,10} might help overcome these difficulties. In this context, DNA has the appropriate molecular-recognition¹¹ and mechanical^{12–16} properties, but poor electrical characteristics prevent its direct use in electrical circuits. Here we describe a two-step procedure that may allow the application of DNA to the construction of functional circuits. In our scheme, hybridization of the DNA molecule with surface-bound oligonucleotides is first used to stretch it between two gold electrodes; the DNA molecule is then used as a template for the vectorial growth of a 12 μm long, 100 nm wide conductive silver wire. The experiment confirms that the recognition capabilities of DNA can be exploited for the targeted attachment of functional wires.

The first step in the construction of the silver wire involves the self-assembly of a DNA template connecting two gold electrodes 12–16 μm apart (see Fig. 1 for an outline of the procedure). First, 12-base oligonucleotides, derivatized with a disulphide group at their 3' end, are attached to the electrodes through sulphur–gold interactions. The electrodes are each marked with specific but different oligonucleotide sequences. A connection is then made by hybridizing two distant surface-bound oligonucleotides with a 16 μm long and fluorescently labelled λ -DNA that contains two 12-base sticky ends, where each of the ends is complementary to one of the two different sequences attached to the gold electrodes. Hybridization on both ends is facilitated by covering the electrodes with an aqueous solution containing the λ -DNA and inducing a flow perpendicular to the electrodes, thereby stretching the λ -DNA molecules in the flow direction (other stretching methods can be used; for application of an electric field, see ref. 17). The flow is terminated when a single DNA bridge is observed by fluorescence microscopy (see Fig. 2), usually after a few minutes. Curving of the

DNA bridge under a flow parallel to the electrodes shows it to be attached solely to the electrodes. The method does not guarantee a single DNA bridge. However, *in situ* video microscopy and imaging of the resulting silver wire by atomic force microscopy (AFM; see below) reveal a silver bridge only in places where DNA was previously fluorescently imaged. We also tried stretching the DNA between two electrodes in the reverse order, performing hybridization and ligation of the disulphide-derivatized oligonucleotides to the long DNA molecule before it was applied to the sample (see Methods section). The binding of the derivatized λ -DNA in this case was again aided by an induced perpendicular flow. Both methods work equally well.

To instill electrical functionality, silver metal is vectorially deposited along the DNA molecule. The three-step chemical deposition process (see Fig. 1 and Methods) is based on selective localization of silver ions along the DNA through Ag^+/Na^+ ion-exchange¹⁸ and formation of complexes between the silver and the DNA bases^{19–21}. The Ag^+/Na^+ ion-exchange process is monitored by following the almost instantaneous quenching of the fluorescence signal of the labelled DNA. The ion-exchange process, which is highly selective and restricted to the DNA template only, is terminated when the fluorescence signal drops to 1–5% of its initial value (the quenching is much faster than normal bleaching of the fluorescent dye). The silver ion-exchanged DNA is then reduced to form nanometre-sized metallic silver aggregates bound to the DNA skeleton. These silver aggregates are subsequently further ‘developed’, much as in the standard photographic procedure, using an acidic solution of hydroquinone and silver ions under low light conditions^{22,23}. Such

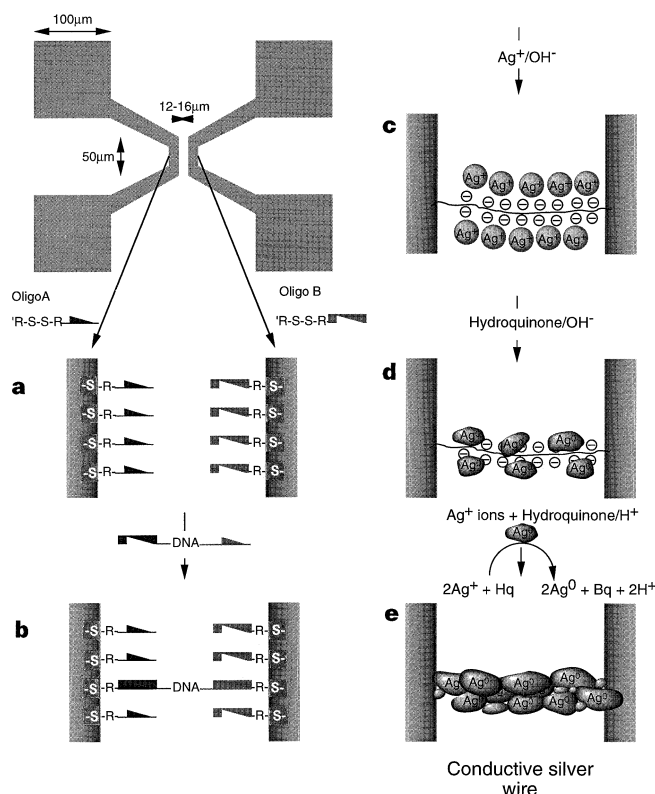


Figure 1 Construction of a silver wire connecting two gold electrodes. The top left image shows the electrode pattern ($0.5 \times 0.5 \text{ mm}$) used in the experiments. The two $50 \mu\text{m}$ long, parallel electrodes are connected to four ($100 \times 100 \mu\text{m}$) bonding pads. **a**, Oligonucleotides with two different sequences attached to the electrodes. **b**, λ -DNA bridge connecting the two electrodes. **c**, Silver-ion-loaded DNA bridge. **d**, Metallic silver aggregates bound to the DNA skeleton. **e**, Fully developed silver wire. A full description of the preparation steps can be found in the Methods section.

solutions are metastable, and spontaneous metal deposition is normally very slow. However, the silver aggregates on the DNA act as catalysts and significantly accelerate the process. Under the experimental conditions, metal deposition therefore occurs only along the DNA skeleton, leaving the passivated glass practically clean of silver. The silver deposition process is monitored *in situ* by differential interference contrast (DIC) microscopy and terminated when a trace of the metal wire is clearly observable under the microscope. The metal wire follows precisely the previous fluorescence image of the DNA skeleton. The structure, size and conduction properties of the metal wire are reproducible and dictated by the 'developing' conditions.

AFM images of segments of a 100 nm wide, 12 μm long wire connecting the two gold electrodes are presented in Fig. 3. The images are representative for the whole wire. As clearly seen, the wire consists of grains of 30–50 nm diameter deposited along the DNA skeleton. We have also fabricated wires as narrow as 25 nm but AFM imaging showed the silver coating to be discontinuous, with some gaps between silver grains.

Two-terminal electrical measurements were made on the silver wire shown in Fig. 3. The resultant I – V curves, obtained with an apparatus having an internal resistance of $\geq 10^{13} \Omega$, are displayed in Fig. 4. The curves are highly nonlinear and asymmetric with respect to zero bias. The shape of the curves depends on the voltage scan direction indicated by arrows. Approaching zero voltage from large positive or negative bias, a zero-current plateau develops with differential resistance larger than $10^{13} \Omega$. At a higher bias, the wire again becomes conductive with a differential resistance somewhat lower than in the original bias polarity. Repeated measurements of a given wire, in the same scan direction, yield reproducible I – V curves.

The origin of the extremely high resistance at small bias, and of the dependence of the current on the voltage scan direction, are not yet clear. If one invokes the Coulomb blockade phenomenon and the grain charging energy, ~ 0.1 eV, as inferred from the grain size in the AFM images, the large plateau requires simultaneous charging of a large number of grains in series. It is not clear, however, whether such a mechanism can yield a history-dependent I – V curve. Another source of nonlinearity might be inter-grain boundary resistance due to silver corrosion. The shape of the I – V curve could then be attributed to electrochemical processes in the corrosion barriers.

The length of the zero bias plateau in different wires may vary

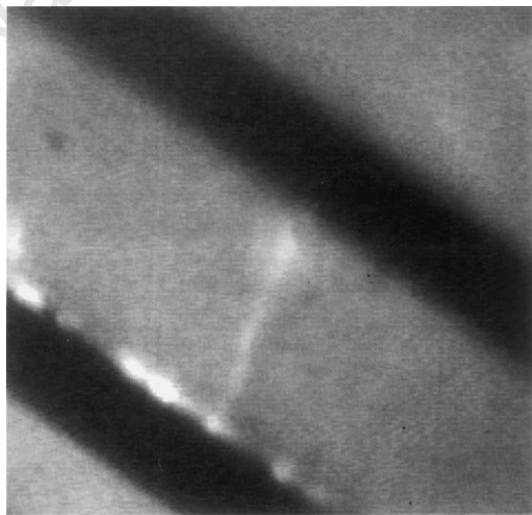


Figure 2 Fluorescence image of the DNA bridge. Fluorescently labelled (Yoyo-1, Molecular Probes, Eugene, Oregon) λ -DNA is stretched between two gold electrodes (dark strips), 16 μm apart. The electrodes are connected to large bonding pads 0.25 mm away.

from a fraction of a volt to roughly 10 V, depending on the silver growth conditions. The solid line in Fig. 4b depicts, for example, the I – V curve of a different wire in which the silver growth on the DNA was more extensive. Compared with the first wire, the plateau was reduced from about 10 V to 0.5 V, and the differential resistance at voltages beyond the plateau was reduced from 30 M Ω to 7 M Ω . By applying voltages higher than about 50 V to the wires that went through extensive silver deposition, the plateau could usually be permanently eliminated to give ohmic behaviour (dashed-dotted line in Fig. 4b).

Each experiment was accompanied by two control measurements to investigate whether either the DNA bridge or the deposited silver on their own could contribute to the observed electrical transport. The two insets in Fig. 4b show the I – V curves obtained for a λ -DNA bridge in the absence of silver deposition (bottom inset) and for a neighbouring device without a DNA bridge that underwent the full silver deposition treatment (top inset). Both control measurements consistently yielded a resistance higher than our measurement capabilities, $10^{13} \Omega$. (Note that the current scale in both insets is 100 times smaller than in the main graph.) Our direct d.c. measurements showed the 16 μm long double-stranded DNA to be practically insulating. It is not clear how to compare our measurements with previously published electron-transfer rates obtained from optical measurements on short DNA segments^{24–26}. Perfect correlation was found between the appearance of a DNA bridge in the fluorescence image, the formation of a silver wire connecting the

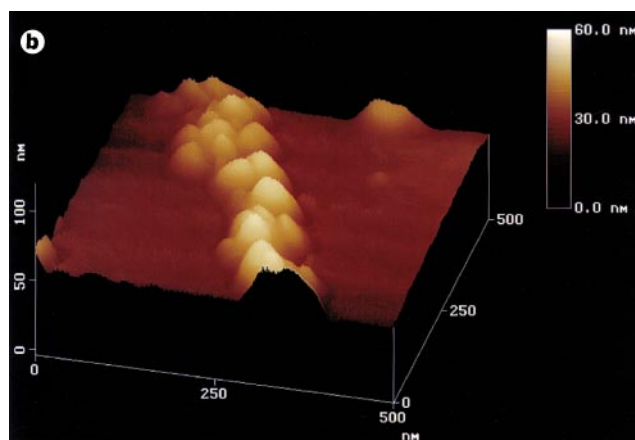
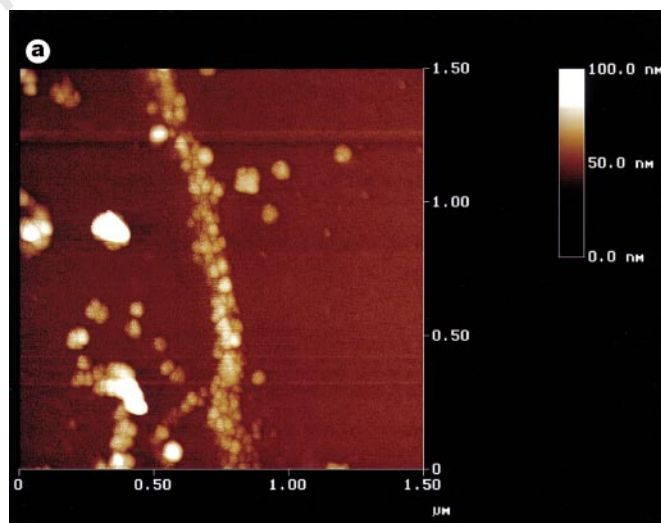


Figure 3 Atomic force microscopy images (Dimension 3000, Digital Instruments) of a silver wire connecting two gold electrodes 12 μm apart. **a**, 1.5 μm ; **b**, 0.5 μm field sizes. Note the granular morphology of the conductive wire.

electrodes, and the resulting conduction between the electrodes. These observations, in conjunction with the control experiments, prove that the electric current is carried solely by the silver deposited on the DNA bridge.

The remarkable recognition capabilities of DNA, used in this study to construct a metal wire connecting two electrodes, have also been exploited in other recent studies. The use of DNA has, for example, allowed researchers to organize colloidal particles into macroscopic crystal-like aggregates^{27,28} and to dictate the shape and size of semiconductor nanoparticle assemblies²⁹. The use of a DNA polyanion as a template for the assembly of electronic materials is not limited to metals. In another study, we have used a DNA template to fabricate a poly-(*p*-phenylene vinylene) (PPV) filament by attaching a positively charged *pre*-PPV polymer to the stretched DNA and subsequently treating it to form a highly photoluminescent PPV wire³⁰.

The construction of functional nanoscale electronic devices is likely to require self-assembly processes. The present study provides a step towards such developments: we have demonstrated that DNA is a sophisticated substrate for the targeted attachment of a conductive metal wire whose width is well below the minimal dimensions accessible by standard, large-scale microelectronics technology. Even though the hysteric *I*-*V* curves that we observe are interesting, low-resistance, ohmic metal wires are essential for

future applications. Such wires might be accessible by making use of different growth conditions, different metal types or post-growth thermal treatments. □

Methods

Sample preparation. A glass coverslip is immersed in fuming nitric acid for 10 min, rinsed with DI water, immersed in a 1M NaOH solution for a further 10 min and rinsed again with DI water. The cleaned glass is dried thoroughly and then passivated against spurious DNA binding by immersion for 12 hours in a 1:5 v/v solution of trimethyl chlorosilane (Sigma) in tetrachloroethane (Sigma). The sample is then rinsed carefully several times with tetrachloroethane and isopropanol and dried thoroughly. Electrodes, 12 μm or 16 μm apart, are defined on the coverslip by standard photolithography and subsequent high vacuum deposition of a 50 nm gold layer on top of a 10 nm titanium adhesion layer. A lift-off process then follows.

Constructing the DNA bridge (see Fig. 1). One gold electrode is wetted with a 10^{-4} μl droplet of an aqueous solution containing 0.2 nM of a 5'-GGGCGGCGACCT-3'-disulphide oligonucleotide (Oligo A) and 10 mM NaCl. Similarly, the other electrode is marked with a solution containing 5'-AGGTCGCCGCC-3'-disulphide oligonucleotides (Oligo B). Both oligonucleotides are synthesized using a 3'-C6-disulphide CPG (Clontech Laboratories, Palo Alto, California). After rinsing, the structure is covered by a 100 μl solution of λ -DNA (0.2 pM, Promega, Madison, Wisconsin) in 10–100 mM NaCl. The λ -DNA has two sticky ends that are complementary to Oligos A and B. A flow perpendicular to the electrodes is induced by micropipette suction of the solution. The flow stretches the λ -DNA molecules perpendicular to the electrodes, leading to their hybridization with Oligos A and B attached to the two electrodes.

Hybridization and ligation before application to the electrodes. The two types of oligonucleotides are phosphorylated at their 5' ends using T4 polynucleotide kinase (New England Biolabs, Beverly, Massachusetts). The 5' phosphate of the λ -DNA is removed using Shrimp alkaline phosphatase (Amersham, Arlington Heights, Illinois) to prevent ligation of its complementary sticky ends to form a cyclic DNA. One type of oligonucleotide is then hybridized with the λ -DNA (10^3 -fold excess of oligonucleotides) while the solution (sodium phosphate buffer and 1M NaCl, pH = 7) slowly cools down (16 hours) from 75 °C to 4 °C. The hybridization is followed by ligation using T4 ligase (New England Biolabs) at 16 °C for 16 hours. The solution is then filtered (Microcon-100, Amicon, Beverly, Massachusetts) to remove excess free oligonucleotides. The second type of oligonucleotide is then hybridized and ligated with the other sticky end of the λ -DNA using the same procedures.

Silver deposition. Being a polyanion, the DNA bridge is loaded with silver ions by Na^+/Ag^+ ion exchange using a 0.1M AgNO_3 basic aqueous solution (ammonium hydroxide, pH = 10.5). The silver ion/DNA complex is reduced using a basic hydroquinone solution (0.05M, ammonium hydroxide, pH = 10.5) to form small metallic silver aggregates bound to the DNA skeleton. The DNA templated wire is 'developed' using an acidic solution (pH = 3.5, citrate buffer) of hydroquinone (0.05M) and silver ions (0.1M) under low light conditions to give a silver wire along the DNA skeleton.

Imaging setup. An inverted microscope (Axiovert 135, Zeiss), equipped with 100 $\times$ oil-immersed objective, 100-W mercury lamp with filters, image intensifier (C-2400 Hamamatsu) and video processor (Omnex, Imagen Instrumentation), is used for fluorescence imaging of the DNA molecules. The same microscope and objective, with the proper DIC slide, and a 100-W halogen lamp are used for DIC imaging of the silver wire.

Electrical measurements. All electrical measurements were carried out between two bonding pads (see Fig. 1) using a 4145 HP parameter analyser with internal resistance $\geq 10^{13} \Omega$ and current resolution of 10 fA.

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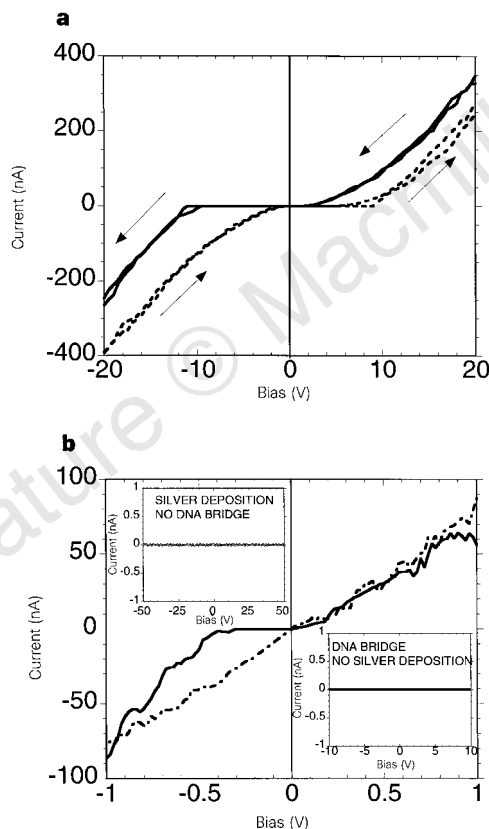


Figure 4 Experimentally observed *I*-*V* curves. **a**, Two terminal *I*-*V* curves of the silver wire shown in Fig. 3. Arrows indicate voltage scan direction. The two curves in each direction present repeated measurements thus demonstrating the stability of a given wire. Note the different asymmetry pertaining to the two scan directions. **b**, *I*-*V* curves of a different silver wire in which the silver growth was more extensive than in **a**. Extensive growth resulted in a narrower current plateau (solid curve), on the order of 0.5 V, and a lower differential resistance (7 M Ω versus 30 M Ω in **a**). By applying 50 V to the wire, the plateau has been permanently eliminated to give an ohmic behaviour (dashed-dotted line), over the whole measurement range. *I*-*V* curves of a DNA bridge with no silver deposition, and silver deposition without a DNA bridge, are depicted in the bottom and top insets, respectively. In both cases, the sample is insulating.

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Correspondence should be addressed to E.B. (erez@physics.technion.ac.il).

Breakup and conditions for stability of the northern Larsen Ice Shelf, Antarctica

C. S. M. Doake*, H. F. J. Corr*, H. Rott†, P. Skvarca‡ & N. W. Young§

* British Antarctic Survey, Madingley Road, Cambridge CB3 0ET, UK

† Institut für Meteorologie und Geophysik, Universität Innsbruck, Innrain 52, A-6020 Innsbruck, Austria

‡ Instituto Antártico Argentino, Cerrito 1248, 1010 Buenos Aires, Argentina

§ Antarctic CRC and Australian Antarctic Division, GPO Box 252–80 Hobart, Tasmania 7001, Australia

The breakup of ice shelves has been widely regarded as an indicator of climate change¹, with observations around the Antarctic Peninsula having shown a pattern of gradual retreat, associated with regional atmospheric warming and increased summer melt and fracturing processes^{2–9}. The rapid collapse of the northernmost section of the Larsen Ice Shelf (Larsen A), over a few days in January 1995, indicated that, after retreat beyond a critical limit, ice shelves may disintegrate rapidly. Here we use a finite-element numerical model that treats ice as a continuum without fracture¹⁰ to examine the breakup history² between 1986 and 1997 of the two northern sections of Larsen Ice Shelf (Larsen A and Larsen B), from which we establish stability criteria for ice shelves. Analysis of various ice-shelf configurations reveals characteristic patterns in the strain rates near the ice front which we use to describe the stability of the ice shelf. On Larsen A, only the initial and final ice-front configurations show a stable pattern.

Larsen B at present exhibits a stable pattern, but if the ice front were to retreat by a further few kilometres, it too is likely to enter an irreversible retreat phase.

Our finite-element model is based on one described by MacAyeal and Thomas¹¹ that was used to analyse strain-rate patterns on the Filchner Ronne Ice Shelf¹². A similar model has been used by C. Hulbe (available on the World-Wide Web at <http://student-www.uchicago.edu/users/hulbe/science/larsen/larsen.html>) to examine the stability of the geometry of the Larsen Ice Shelf after the iceberg calving in 1995. We used the model in a time-independent (diagnostic) mode in which the only input data required are ice thickness and grounding-line velocity. Ice thickness data (Fig. 1) were taken from a mixture of airborne radar ice thickness measurements (made in 1995–96) and surface elevation data from satellite altimetry. Velocities measured in the interior of the ice shelf are too sparse to be used for constraining the ice viscosity through control methods¹³. Measurements that are available^{7,14} are used as checks to ensure that modelled velocities conform to realistic values. Except for a softening at the nodes around the margin to one-quarter stiffness, the ice stiffness was constant everywhere, which is equivalent to assuming a uniform depth averaged temperature throughout the ice shelf. Thus the model is of a homogeneous ice shelf and ignores the complexity present in the crevasses and rifts.

We analysed each ice-shelf configuration for its instantaneous strain-rate pattern. We hypothesized that characteristic patterns will emerge that can be associated with the known history and behaviour of the ice shelf. These findings are then used to predict the likely behaviour of Larsen B, at present the northernmost ice-shelf section, under its present circumstances. We assume that a ‘stable’ ice-front position, although possibly experiencing retreat, is capable of readvancing, but that an ‘unstable’ position is likely to undergo irreversible retreat. The process of (re)formation of ice shelves is probably very different from their disintegration, with timescales estimated to be of the order of centuries. The complex processes of fracturing and rifting of the ice shelf in response to the flow field and external climatic forcing, although important to the retreat, are poorly known and are not included in our model. We believe the indicators derived from the characteristic patterns are robust under these simplifications. Thus we do not attempt to predict the rate of ice-front retreat or where or when calving will take place, but do indicate the possible consequences if it were to happen.

Dates for the ice-front positions analysed are shown in Fig. 2. The earliest dates used, 1986–89, represent a marginally stable situation for Larsen A and a stable one for Larsen B⁶. The rapid changes taking place on Larsen A, especially around January 1995 when a catastrophic collapse occurred², means that several positions were analysed that were close together in time. For Larsen B, only two ice-front positions have been analysed, one for the ‘stable’ situation before the iceberg calving, also in January 1995, and the other the position in March 1997. There has been continual retreat along most of the ice front since the iceberg calving event, at a rate of a few kilometres per year, with no sign that the trend is about to cease or be reversed⁷.

Preliminary model runs using the 1986–89 ice-front positions were carried out using several ice viscosities to find a fit to the observed velocities. The velocities on Larsen B were measured over a two-year period, 1994–96, and so cover a number of ice-front configurations. There was no attempt to find the best fit to the velocities, just a reasonable match, because of the paucity of velocity data and the approximation of uniform viscosity. The chosen viscosity value was used for all ice-front positions. Model runs performed with ice up to 15% thinner near the ice front to test the sensitivity to thickness showed the amplitude of the strain rates there decreased by up to 50% but without any significant change in the pattern. One implication is that ice shelves are more sensitive to climate change through fracture than through thinning.

Model results show that there are large changes in the Larsen A

Figure 1 Location diagram and ice-thickness contours. Surface elevations (m) from Seasat (1978), Geosat (1987) and ERS (1993–95) altimetry data were fitted to a common geoid and compared with radar thickness data to obtain a best (linear) fit assuming the ice was floating in hydrostatic equilibrium in the sea. Thickness data were input to the model using a grid with 3 km spacing. Because the thickness data were obtained over a long time period, we used the same ice-thickness distribution for each ice-front position. Justification is provided by airborne radar ice-thickness measurements made in February 1975 near Robertson Island which show good agreement with the 1995–96 values, suggesting that, despite the dramatic loss of ice shelf in the intervening period, there has been relatively little change in the ice-thickness distribution in the remaining shelf.

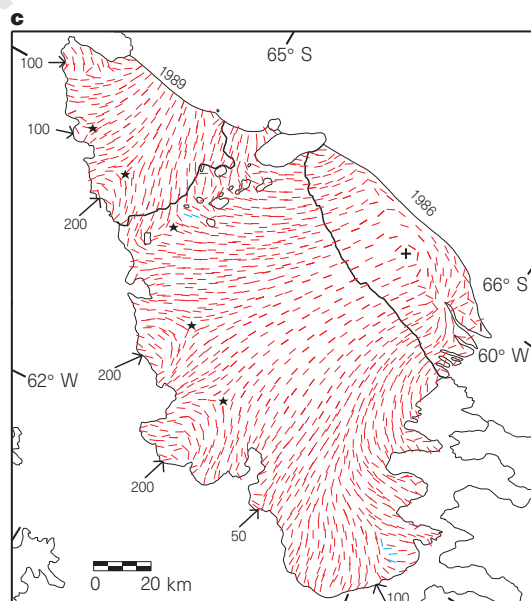
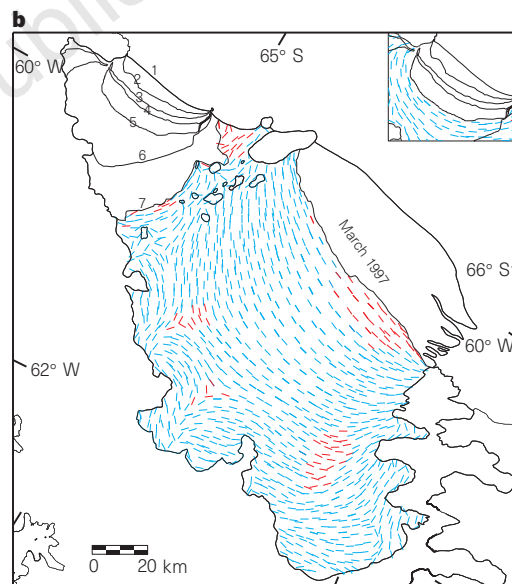
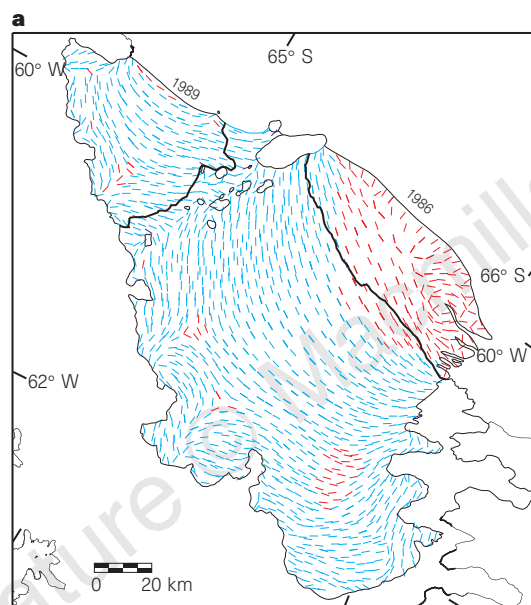
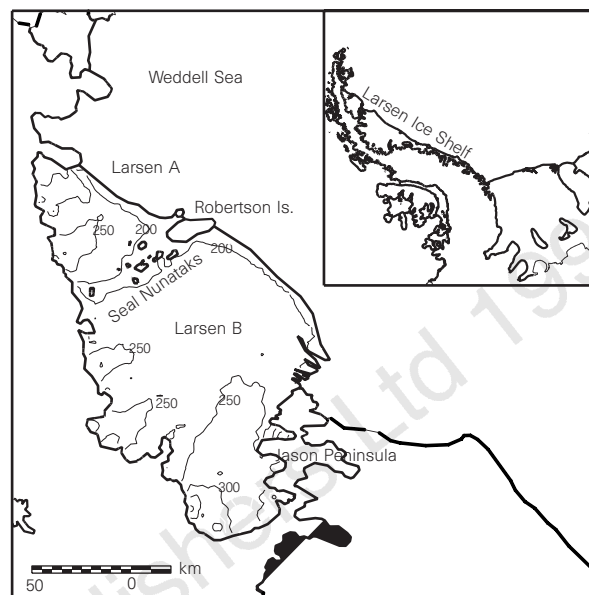


Figure 2 Strain-rate trajectories. **a, b**, Strain-rate trajectories (ϵ_2) for the ice-front configuration on 1986–89 (**a**) and March 1995–97 (**b**) (inset at top right shows the configuration for Larsen A on 25 January 1995, at the same scale). Red lines indicate extension, blue compression. **c**, Strain-rate trajectories (ϵ_1) for the ice-front configuration on 1986–89 (together with isotropic points and glacier input velocities (m yr^{-1})). Positions and dates of the ice fronts used in the model runs are identified by their model run number (**b**). The Larsen A ice-front dates used for model runs 1–7 were 1989, 8 December 1992, 12 January 1993, 16 February 1993, 25 January 1995, 30 January 1995 and 22 March 1995. For Larsen B the ice-front date used for model runs 1–5 was 1986 and the date used for model runs 6 and 7 was 21 March 1997. The full date is given when satellite imagery was used to determine the ice-front position⁶, and the year-only date indicates that the position is taken from the Antarctic Digital Database²⁰.

strain-rate trajectories (direction) and magnitudes as the ice front retreats and alters the bay geometry. Although the trajectories for Larsen B are not changed significantly by the iceberg calving, small changes in the strain-rate magnitudes are seen as there is then less side-wall restraint for Robertson Island.

Analysis of the strain rates for different ice-shelf configurations (Fig. 2) shows the characteristic patterns of a 'compressive arch' and of isotropic points which were previously identified in the study of the Filchner Ronne Ice Shelf¹². The 'compressive arch' is observed in the strain rates for the least principal component (ϵ_2). Seaward of the arch, both principal strain-rate components (ϵ_1 and ϵ_2) are extensive, whereas inland the ϵ_2 component is compressive. The arch extends from the two ends of the ice front across the whole width of the ice shelf. It is a generic feature of ice shelves studied so far and is structurally stable to small perturbations¹². It is probably related to the geometry of the ice-shelf bay, and may be of broader interest for studies of other ice shelves and their interactions with climate. We suggest that a critical arch, consisting entirely of compressive trajectories, corresponds to a criterion for stability. If the ice front breaks back through the arch then an irreversible retreat occurs, possibly catastrophically. We cannot yet determine in advance the location of the critical arch, but it is probably close to the transition from extension to compression for ϵ_2 .

The trajectory (direction) and sign (extensive or compressive) of ϵ_2 are shown in Fig. 2a, b. For Larsen A there were strain rates of both signs along the ice front in 1989 (Fig. 2a), but in subsequent positions the strain rates near the ice front were all compressive until March 1995 (Fig. 2b), when again strain rates of both signs are found. By this time the only ice shelf left was a section joining the almost stagnant ice around Seal Nunataks and a small area north of Robertson Island⁷ where the strain rates were all extensive (Fig. 2b) and which disappeared in 1996. This behaviour gives a strong indication that once a retreating ice front breaks through the critical 'compressive arch' then retreat is irreversible. Thus, if strain rates at the ice front indicate that its position is seaward of the 'compressive arch' (that is, there are some extensive values for ϵ_2) then the ice shelf may be in a stable condition. If, however, all the values for ϵ_2 are compressive, indicating that the ice front is inland of the 'compressive arch', then the ice shelf may be undergoing unstable retreat. On Larsen A in late 1994, just before its collapse, large-scale surface undulations, rifts and other features were observed where it had previously been almost ideally flat⁶. This may be an important clue in understanding the detailed breakup process.

Isotropic points exist near the input glaciers, and near the ice front if it is in a stable configuration. These are indicators of generic features in the surface flow field that are stable to small perturbations in the flow^{15–19}. Thus, their existence should not be sensitive either to reasonable errors in the data used in the model or to simplifications in the model itself. They act as (permanent) markers in a complicated (tensor) strain-rate field and are often located close to points where the two principal strain rates are equal or where the velocity field is stationary (usually either a maximum or a saddle point¹⁸). Isotropic points are classified by several properties, but only two categories can be reliably distinguished observationally, by the way the trajectory of either of the principal strain rates varies around a path enclosing the isotropic point. In one case the trajectory varies in a prograde sense and the isotropic point is called a 'lemon' or 'monstar'; in the other case the trajectory varies in a retrograde sense and the isotropic point is called a 'star'. We can identify the two categories in the model strain-rate trajectories, with stars occurring near input glaciers and monstars or lemons near ice fronts. (In practice it is impossible to distinguish between monstars and lemons at the resolution of the data or model, so we shall henceforth call them monstars.) Icebergs calving off the Filchner Ice Shelf in 1986 moved the position of the monstar up to the newly formed ice front¹², whereas on the Ronne Ice Shelf the monstar is about 50 km behind the ice front. This suggests that a

monstar can be used as a 'weak' indicator of a stable ice front. Calving can remove a monstar even if the subsequent ice-front position is not necessarily unstable and may readvance.

The trajectory of the greatest principal strain rate (ϵ_1) is positive (extending flow) over nearly all the ice shelf in the 1986–89 configuration (Fig. 2c). The pattern shows isotropic points (stars) near where glaciers enter the ice shelf and, for Larsen B, there is a monstar near the ice front. Stars are seen for all the different ice-front configurations, indicating that no fundamental change is occurring near the grounding line as a result of the ice front retreating. A monstar is seen for Larsen B only before the iceberg calved in 1995. The disappearance of the isotropic point is perhaps a weak indication that the iceberg calving event was greater than expected for normal calving from a stable ice shelf, and suggests that further retreat may be expected. The lack of a monstar for Larsen A in 1989 suggests that the relatively slow ice-front retreat observed before then had already broken back beyond where the isotropic point presumably once existed when the ice shelf was in a more stable configuration.

For Larsen B, the iceberg that calved in 1995 removed the isotropic point but did not break through the compressive arch. (We note that the highest principal stresses in the model were concentrated along the iceberg calving line.) However, the subsequent retreat of a few kilometres per year has brought the Larsen B ice front close to the arch, and if retreat continues at the same rate for the next few years it will be breached. We suggest that, if that happens, Larsen B will decay until a new stable position is reached much closer to the mainland. We do not know how quickly the decay will occur, nor whether it will follow either the final catastrophic pattern shown by Larsen A or the slower disintegration pattern shown by the Wordie Ice Shelf⁸. However, unless the situation changes dramatically and ice-front retreat ceases almost immediately, it seems fairly certain that another ice shelf will disappear, perhaps even this century. □

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Correspondence should be addressed to C.S.M.D.

Composition of fluids in the lower crust inferred from metamorphic salt in lower crustal rocks

Gregor Markl & Kurt Bucher

Institut für Mineralogie, Petrologie und Geochemie, Albert-Ludwigs-Universität, Albertstrasse 23b, 79104 Freiburg, Germany

Knowledge of the rheological properties of the lower crust and the metamorphic processes that operate there is important for our understanding of orogenic processes and granite genesis. The rheological properties critically depend on whether fluids are present in the lower crust^{1,2} and, if present, on their composition^{3–6}. Fluid-inclusion^{7–9} and phase-equilibria^{4,10} studies of lower crustal granulites have shown that fluids with low water activities (due to the presence of dissolved components such as CH₄, N₂, CO, CO₂ and chlorides)¹¹ are present at least episodically in the lower crust. Here we report the occurrence of a solid salt solution (NaCl–KCl) found together with chlorine-rich amphibole and biotite in lower crustal granulites. A desiccation mechanism explains how salt and chlorine-rich minerals formed from an originally water-rich fluid through a short-lived series of hydration reactions in the granulites, during which chlorine was progressively enriched in the fluid. Consequently, it would appear that fluid was present in the lower crust in only small

amounts and was not stable over geologically long periods of time, leading to the conclusion that the lower crust is devoid of a free fluid phase during most of its history.

The Lofoten Islands of northern Norway are a typical example of a Proterozoic granulite facies terrain with minor retrogression. They consist of metavolcanic and metasedimentary gneisses intruded by dry granitic melts (mangerites and charnockites), anorthosites and gabbros around 1.75 Gyr (ref. 12). At this time, the area experienced granulite-facies conditions of ~750–800 °C at 4 kbar. In the tectonic environment of crustal thickening after the intrusive activity, only minor amounts of fluids infiltrated the anhydrous pyroxene–olivine–feldspar rocks on Lofoten and resulted in the formation of variably Cl-enriched hydrosilicates (amphibole and biotite) and salt (halite–sylvite solid solution) in small amounts on the whole archipelago under pressure–temperature conditions of ~600 °C and 8–11 kbar. Eclogite-facies assemblages show the culmination of crustal thickening at 700 °C and >14 kbar (ref. 13). Later amphibolite facies overprint at Grenvillian (1.1 Gyr) and Caledonian (460–350 Myr) times^{12,14} resulted only in incomplete retrogression of earlier high-temperature and high-pressure assemblages.

For the detection of the salt, the following technique was used: because salt is water-soluble, the samples were crushed or sawed slowly without water, and a small piece was immediately carbon-coated and loaded into a scanning electron microscope (SEM) or into an electron microprobe. Then, the samples were scanned for increased chloride concentrations using energy-dispersive spectrometry (EDS) or wavelength-dispersive spectrometry (WDS). This technique is considerably faster and more simple than previously used techniques¹⁵ and allows quick assessment of whether salt is present in a sample or not.

Salt was found in three samples (out of ten that were investigated) from two different rock types. Samples GM 93 and GM 96 came from a shear zone in an anorthosite near Nusfjord on Flakstadøy; sample GM 402 came from a charnockite pegmatite near Hopen on Austvågøy. All three samples contain garnet, clinopyroxene, plagioclase and highly Cl-enriched amphiboles. GM 93 and GM 96 additionally bear Cl-rich biotite, kyanite and scarcely quartz and scapolite; GM 402 contains K-feldspar and abundant quartz. Combination of Grt–Bio thermometry corrected for the effect of Cl and Ti in biotite¹⁶ with the garnet–plagioclase–kyanite–quartz equilibrium indicates that the Cl minerals in samples GM 93 and GM 96 formed at ~550–620 °C and at a pressure of 8–11 kbar (corresponding

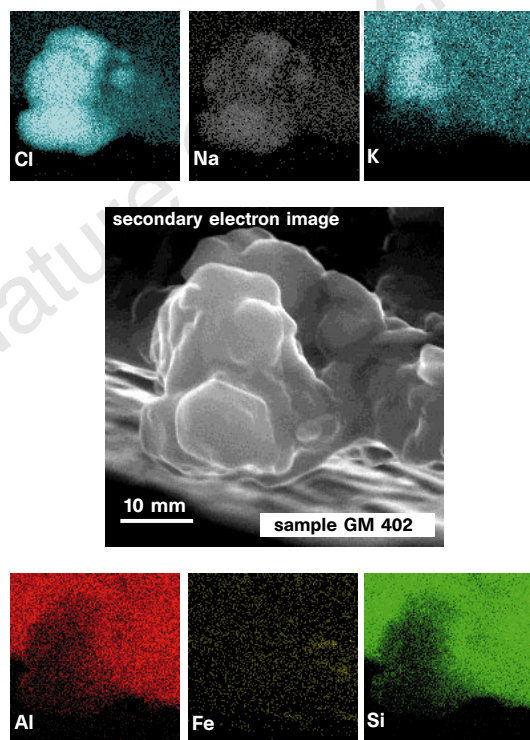


Figure 1 Secondary electron image and microprobe element maps of a typical aggregate of metamorphic salt in sample GM 402. The microprobe element maps show the distribution of Cl, Na, K, Al, Fe and Si in the same area as the secondary electron image. The presence of KCl- and NaCl-dominated portions in the salt aggregate is evidence of a primary high-temperature (Na, K)Cl solid solution that exsolved during cooling. Low-temperature salt (resulting from contamination, for example) would not show this feature.

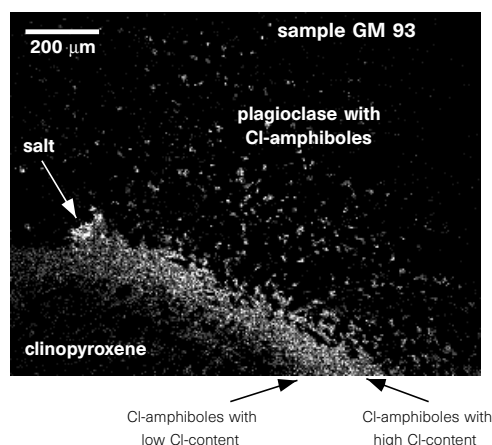


Figure 2 Microprobe element map showing the distribution of Cl in a portion of sample GM 93. The lighter a particular point is, the higher is the Cl content at this point. In the lower left corner, a grain of cpx (black, as it contains no Cl) is surrounded by progressively more Cl-rich (lighter) amphiboles. The outermost amphiboles are the richest in Cl and coexist with salt. This pattern supports the suggested desiccation mechanism described here for the formation of the salt.

to 25–35 km depth). The uncertainties represent the whole range in measured mineral compositions and a range in silica activities (a_{SiO_2}) between 0.6 and 1. The salt is present on grain boundaries and its size ranges from 5 to 50 μm . It cannot be derived from a fluid inclusion cracked open during sample preparation because fluid inclusions are completely absent in these rocks. Figure 1 shows a back-scattered electron image and element distribution maps of a high-temperature, super-solvus salt, which is now exsolved to halite (NaCl) and sylvite (KCl).

The origin of the salt is related to desiccation of a fluid phase and formation of the Cl-rich amphibole and biotite. Cl-amphibole and Cl-biotite form layers around clinopyroxene and Fe–Ti oxides. Textures prove that they formed during one tectonometamorphic event, but the Cl content of the amphiboles steadily increases from the inner to the outermost part of this layer (Fig. 2), indicating changes in fluid composition during the presumably isothermal and isobaric growth of the minerals. Crystal chemical controls^{17,18} alone are not able to explain the observed composition variations¹⁹. In Fig. 2, salt is found in the outermost parts of the layer together with the most Cl-enriched amphibole. Cl-poor minerals are considerably more common than Cl-rich minerals (see distribution of Cl content in amphibole in sample GM 96 in Fig. 3). Hence, the salt formed during the final stages of desiccation of a water-rich fluid that infiltrated the lower crustal rocks. As suggested in refs 16 and 19, the changing Cl content of amphiboles and biotite record this process. Principal component analysis^{16,19} and the calculation of $\text{H}_2\text{O}/\text{HCl}$ fugacity ratios from biotite compositions²⁰ support this interpretation.

The desiccation process can be modelled by assuming a Cl-poor

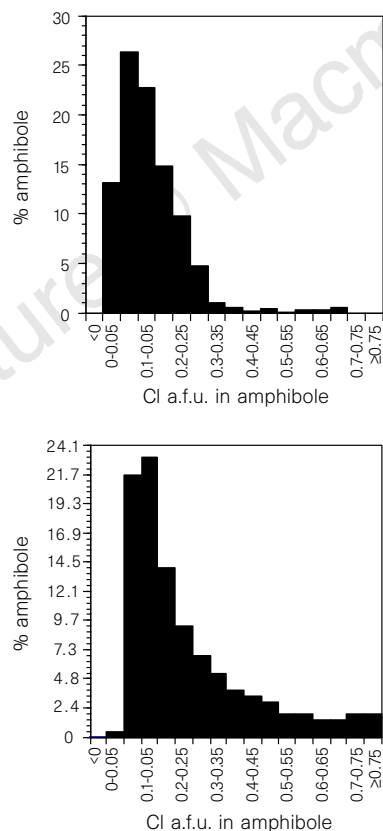


Figure 3 Measured (top) and calculated (bottom) distribution of Cl in amphibole from sample GM 96 (a.f.u., atoms per formula unit). These results support the proposed desiccation mechanism: the frequency distribution calculated from the equations given in the text is very similar to the measured frequency distribution ($n = 952$). If the various Cl contents in amphibole were caused by measurement uncertainties, a gaussian distribution would result rather than the asymmetric shape shown here (top).

initial fluid infiltrating the rock and subsequent hydration reactions of the pyroxenes and Fe–Ti oxides which led to the formation of amphibole and biotite. Because of the preferential incorporation of H_2O compared to Cl into these minerals, the fluid will successively become more Cl-rich until it finally reaches saturation with the halite–sylvite solid solution. The reactions taking place can be represented by the model reaction: clinopyroxene + ilmenite + anorthite + fluid = amphibole + biotite + garnet + kyanite + quartz.

In the absence of fluid inclusions from which the fluid composition could be determined, the fluid was treated as a first-order approximation as a binary H_2O –NaCl fluid. This simplification is justified by the complete lack of carbonates, interpreted to reflect a basically CO_2 -free fluid, and by calculations that indicate NaCl/KCl ratios of between 7 and 16 in the evolving fluid. Then, the reaction: OH-biotite + 2 halite + 3 anorthite + 4 quartz = Cl-biotite + 2 albite + grossular + kyanite + H_2O can be used in connection with experimental data^{21–23} and appropriate activity-composition relationships (garnet²⁴, plagioclase²⁵, halite²¹ and H_2O ²¹) to calculate the equilibrium fluid composition (in terms of $X_{\text{H}_2\text{O}} = \text{H}_2\text{O}/(\text{H}_2\text{O} + \text{Cl})$, that is, the salinity as NaCl equivalent concentration) and the fluid evolution. In samples GM 92, 93 and 96, the activity for kyanite was unity (standard state is the pure substance at p and T) and a_{SiO_2} was defined by the grossular–anorthite–kyanite equilibrium. In sample GM 402, a_{SiO_2} was 1 and the activity of kyanite was defined by the grossular–anorthite–quartz equilibrium. For biotite, ideal site mixing was assumed. The calculated Cl distribution in amphibole is very similar to the measured one (Fig. 3). The calculated fluid compositions range from an $X_{\text{H}_2\text{O}}$ value of 0.98 to 0.88 in a salt-free sample (GM 92; Fig. 4) and from 0.88 to 0.59 and 0.54 in the salt-bearing samples GM 96 and GM 93, respectively. The Cl-richest amphiboles in sample GM 402 define a fluid composition between $X_{\text{H}_2\text{O}}$ values of 0.4 and 0.55 (depending on the equilibrium feldspar composition, which was difficult to determine precisely owing to exsolution). Hence, the halite saturation curve is reached between values of $X_{\text{H}_2\text{O}}$ from 0.6 and 0.5, which is in perfect agreement with experimental data^{21,26} (Fig. 4). In conclusion, initial Cl-poor fluids evolve during their reaction with the granulite-facies mineral assemblage to fluids of high salinity within the halite stability field.

The mechanism of crustal desiccation is expected to have worked

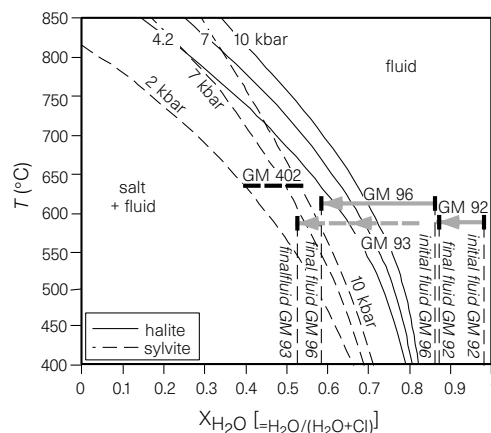


Figure 4 Temperature– $X_{\text{H}_2\text{O}}$ diagram, where $X_{\text{H}_2\text{O}} = \text{H}_2\text{O}/(\text{H}_2\text{O} + \text{Cl})$. In agreement with the suggested desiccation process, the calculated fluid compositional changes (arrows) lead the fluid from initially aqueous compositions into the stability field of salt, which is approximated by the experimentally determined saturation curves for NaCl²¹ and KCl²⁶ in binary aqueous fluids at various pressures and temperatures. The correct position of the saturation surface for the ternary H_2O –NaCl–KCl system is not known. Additional minor components in the fluid, however, would cause the halite saturation to be displaced to lower $X_{\text{H}_2\text{O}}$ values.

in other granulite-facies terrains, too. This is supported by the occurrence of Cl-enriched minerals in many granulite terrains worldwide^{16,27,28}. It is likely that salt will also be found in these granulites. Salt inclusions in mangeritic²⁹ and pantelleritic³⁰ rocks suggest that a salt melt or a highly saline brine may be involved in the generation and the crystallization history of water-undersaturated magmas typical for granulites, by lowering the melting temperature and decreasing the water activity at the same time. On the other hand, the short-lived, episodic occurrence of saline fluids in the lower crust as shown here precludes their general importance for processes that lead to the formation of granulites or melts of low water activities.

Minerals as rich in chloride as those reported here seem to be confined to rocks in which a granulite-facies mineralogy reacted with an aqueous fluid^{19,27,28}. Two exceptions are active hydrothermal systems^{31,32} and metamorphosed evaporite sequences³³. The only occurrence of salt (NaCl–KCl solid solution) in regionally metamorphosed rocks apart from the Lofoten rocks was reported¹⁵ from amphibolite-facies marbles from the Alps, which was attributed to fluid immiscibility. However, the involvement of evaporites in primary chloride enrichment is possible in this case as well.

Cl-rich amphiboles and biotites are found on all Lofoten Islands. Even though salt saturation was demonstrated for only three samples, the Cl-bearing hydrosilicates provide evidence for the desiccation process in the whole Lofoten crustal segment. Consequently, in these lower crustal rocks fluid was not stable and not present during a period as long as 1.4 Gyr apart from very short periods of infiltration. During these periods, the infiltrating fluid was totally consumed as long as the buffer capacity of the granulite-facies assemblages was sufficient. Therefore, fluids are not likely to be present for geologically long periods, particularly in tectonically stable, cratonic areas³⁴. If fluids are present, they would be expected to be in equilibrium with a salt, which can be tested by investigating lower crustal rocks using the technique we describe here. Our discovery of metamorphic salt in partially retrogressed granulites may not be related to its scarcity, but to preparational difficulties with the water-soluble salt. □

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Correspondence should be addressed to G.M. (e-mail: markl@ruf.uni-freiburg.de).

Making mistakes when predicting shifts in species range in response to global warming

Andrew J. Davis*, Linda S. Jenkinson*, John H. Lawton†, Bryan Shorrocks* & Simon Wood††

* Ecology and Evolution Group, Biology Department, The University, Leeds, Yorkshire, LS2 9JT, UK

† NERC Centre for Population Biology, Imperial College Silwood Park, Ascot, Berkshire, SL5 7PY, UK

Many attempts to predict the biotic responses to climate change rely on the ‘climate envelope’ approach^{1–3}, in which the current distribution of a species is mapped in climate-space and then, if the position of that climate-space changes, the distribution of the species is predicted to shift accordingly^{4–6}. The flaw in this approach is that distributions of species also reflect the influence of interactions with other species^{7–10}, so predictions based on climate envelopes may be very misleading if the interactions between species are altered by climate change¹¹. An additional problem is that current distributions may be the result of sources and sinks¹², in which species appear to thrive in places where they really persist only because individuals disperse into them from elsewhere^{13,14}. Here we use microcosm experiments on simple but realistic assemblages to show how misleading the climate envelope approach can be. We show that dispersal and interactions, which are important elements of population dynamics¹⁵, must be included in predictions of biotic responses to climate change.

We designed experiments in which the distribution of species

† Present address: Mathematical Institute, University of St Andrews, North Haugh, St Andrews, Fife, KY16 9SS, UK.

along temperature gradients was first observed in the absence of dispersal and interactions between species. The outcome of these experiments represents the real climate envelope for the species on their own. We then determined how interactions and dispersal distort these envelope distributions by contrasting them with the distributions in clines where both factors acted, and also with those in clines where dispersal, but not interaction, was possible. The interactions studied were between two, three or four species. Finally, we examined the impact of global warming on distributions and abundances in clines subject to both dispersal and interactions.

Our model assemblage contained combinations of three fruitfly species, *Drosophila melanogaster* Meigen, *D. simulans* Sturtevant and *D. subobscura* Collin, and a parasitoid wasp *Leptopilina boulardi* (Barbotin, Carton & Kelner-Pillaut). These species overlap in southern Europe and the Mediterranean basin^{16,17}, and are thus an element of a naturally occurring assemblage. The parasitoid pre-

ferentially oviposits in *D. melanogaster*, in which it suffers little mortality, but it also attacks *D. simulans* and *D. subobscura*, causing high mortality even though the wasp larva rarely survives¹⁸.

We established experimental clines by linking eight cages in series and housing each pair of cages in separate incubators at different temperatures. Each group of four incubators accommodated up to five replicates (Fig. 1), and we set up two such groups. The incubators in a group were sequentially set to 10, 15, 20 and 25 °C ('cold' clines) or, simulating global warming¹⁹, to 15, 20, 25 and 30 °C ('hot' clines).

Competitive interactions in our clines markedly altered the distributions and abundances of all three species from those found in single-species clines (*D. simulans* and *D. subobscura* with one or two competitors; repeated measure analysis of variance (RMA), $F_{1,176} = 177.40$, $P < 0.0001$; *D. melanogaster*, *D. simulans* and *D. subobscura* with two competitors; RMA $F_{2,168} = 237.78$, $P < 0.0001$) (Fig. 2). The species responded differently, with significant interactions of species with number of competitors (two-species interactions RMA, $F_{2,168} = 68.7$, $P < 0.0001$; three-species interactions, $F_{2,168} = 27.32$, $P < 0.0001$). Competitive effects in two-species clines were unbalanced as *D. subobscura* reduced *D. simulans* abundances at only 10 and 15 °C (one-tailed $t = 2.944$, d.f. = 660, $P < 0.01$; $t = 5.33$, d.f. = 665, $P \ll 0.001$, respectively) but *D. simulans* significantly reduced *D. subobscura* abundances at 15 and 20 °C (one-tailed $t = 5.72$, d.f. = 636, $P < 0.001$; $t = 8.45$, d.f. = 636, $P \ll 0.001$, respectively) and also throughout the temperature range (Fig. 2). The proportionately greater reduction in *D. subobscura* at 15 than at 10 °C in this two-species interaction shifted the apparent optimum temperature for *D. subobscura* to 10 °C, away from 15 °C where it had been when *D. subobscura* was on its own. Competition in three-species clines reduced the range of *D. simulans*, which was driven extinct at 10 °C, and of *D. subobscura*, which was driven extinct at 25 °C (Fig. 2). Competition also significantly reduced the abundance of *D. simulans* at all other temperatures (in all cases, one-tailed $t > 9.0$, d.f. = 210, $P \ll 0.001$) (Fig. 2). Our clines show the same strong influence of both the identity and number of competing species that is known in nature^{7,9}. The importance of interactions is that if species respond idiosyncratically to climate change, as the available evidence overwhelmingly suggests^{20–23}, the identity and number of

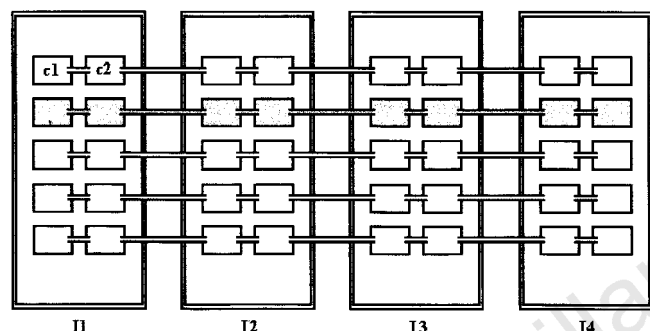


Figure 1 Experimental arrangement. The arrangement of perspex cages (100 × 150 × 300 mm) in one set of four incubators (I1–I4) showing an experimental series of eight cages linked together by tubing (shaded). Each pair of cages (for example, c1–c2) experiences the same temperature.

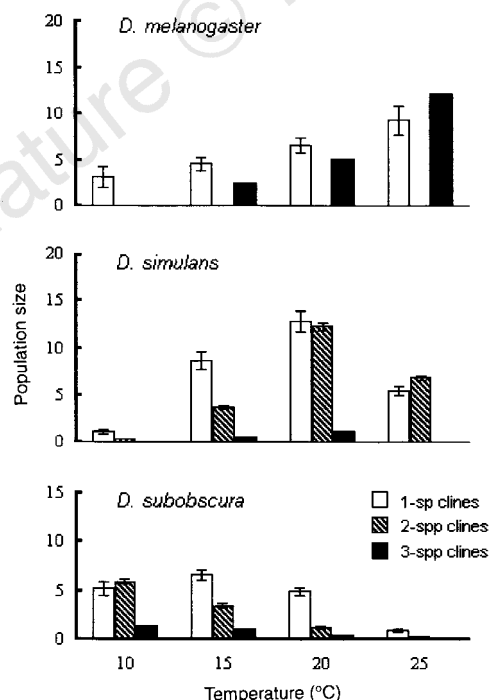


Figure 2 Comparison of *Drosophila* populations in single-species clines, two-species clines, and three-species clines. Two-species clines contained both *D. simulans* and *D. subobscura*, and three-species clines these two species as well as *D. melanogaster*. Error bars, ± 1 s.e. based on means of all measures.

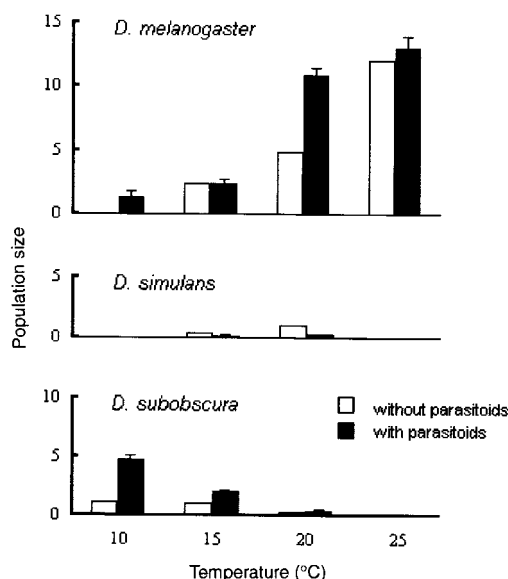


Figure 3 Comparison of *Drosophila* populations in cold three-species clines with or without *L. boulardi* parasitoids. Error bars, ± 1 s.e. based on means of all measures.

competitors will change, and competitive interactions will become uncoupled. Consequently, by ignoring these effects, the climate envelope approach risks making erroneous predictions of range and abundances after climate change.

Adding *L. boulandi* to our three-species clines significantly changed *D. subobscura* populations ($F_{1,180} = 12.40$, $P = 0.003$) and differentially affected the three *Drosophila* species (species and parasitoid interaction RMA $F_{2,216} = 6.09$, $P = 0.003$) (Fig. 3). At 20°C, apparent competition through the parasitoid²⁴ raised the abundance of *D. melanogaster* (one-tailed $t = 7.91$, d.f. = 411, $P \ll 0.001$) (Fig. 3) by decreasing the numbers of the other two *Drosophila*. In contrast, *D. simulans* became vanishingly rare throughout its range (Fig. 3). The wasp increased the abundances of *D. subobscura* at 10°C (Fig. 4) to values close to those reached by *D. subobscura* alone (Fig. 2). *L. boulandi* does not occur at 10°C, so this increase in *D. subobscura* must involve the parasitoid's suppression of competing *Drosophila* elsewhere in the cline, so favouring *D. subobscura* by reducing the numbers of competitors dispersing into the 10°C cages. These findings indicate that the wasp and the *Drosophila* species are involved in complex direct and indirect interactions like those between enemies and victims in natural situations^{8,10,24}. Uncoupling these interactions through divergent species responses to climate change¹¹ will greatly affect the ranges and abundances of species. It is once again evident that interactions will distort, or greatly modify, changes arising directly from climatic change.

In our clines, dispersal significantly altered distributions and abundances from the climate envelope distributions found in its absence (RMA $F_{1,126} = 6.15$, $P = 0.014$) (Fig. 4). Without dispersal, populations at extreme temperatures died out, *D. simulans* and *D. melanogaster* at 10°C and *D. subobscura* at 25°C, temperatures at which populations persisted in open clines. These temperatures are thus outside the physiological climate envelopes of the three species and must be classical sinks¹¹. Dispersal also altered abundances within distributions, lowering *D. melanogaster* and *D. simulans* populations at 20°C (one-tailed $t = 1.89$, d.f. = 216, $P < 0.05$; $t = 3.79$, d.f. = 184, $P \ll 0.001$, respectively) and 25°C (one-tailed $t = 3.34$, d.f. = 242, $P < 0.001$; $t = 1.03$, d.f. = 178, $P < 0.05$, respectively) and *D. subobscura* at 20°C (one-tailed

$t = 3.23$, d.f. = 222, $P < 0.001$). These results demonstrate that dispersal was an important determinant of both distributions and abundances in our model system, just as it is in nature^{14,15}, and markedly modified the simple climate envelope distributions. Dispersal frees local populations from direct dependence on local climate and allows populations to influence each other in metapopulation²⁵ and central-marginal²⁶ networks. Furthermore, as the spatial distribution of interdependent populations changes under global warming²⁷, the pattern and intensity of dispersal will also change. Thus simple climatic extrapolation¹⁻³ will not accurately predict the new ranges and abundances produced by climate change.

We tested the effects of global warming on clines containing interspecific competition and dispersal. Creating hot clines by raising overall cline temperatures by 5°C significantly altered the distribution and abundances of *D. subobscura* in both two-species and three-species clines (RMA $F_{1,30} = 10.44$, $P = 0.003$; $F_{1,54} = 9.49$, $P = 0.003$, respectively) (Fig. 5). Although *D. subobscura* maintained a small population at 25°C in cold clines, its range was reduced in hot clines because it was eliminated at 25°C (Fig. 5) by *D. simulans* or *D. melanogaster* dispersing into the lower temperature from the 30°C zone created in hot clines. Global warming thus drove *D. subobscura* out of the warm, low-latitude, part of its range in which its presence would be predicted from its range when alone. Furthermore, *D. subobscura* abundances within its reduced range also changed markedly under simulated global warming, with higher populations at 15°C in hot clines than at the same temperature in both two-species and three-species cold clines (one-tailed $t = 1.28$, d.f. = 244, $P < 0.05$; one-tailed $t = 5.75$, d.f. = 209, $P < 0.001$) (Fig. 5). This differential change inverted the relative abundances of the species at 15°C, with *D. subobscura* dominating allospecifics in hot clines. If pests were to behave in the same way, a currently unimportant population at 15°C might become economically important at the same temperature under global warming.

These results indicate that global warming can produce 'unexpected' changes of range and abundance in systems incorporating dispersal and species interactions. In the real world, interactions and feedbacks are likely to be even more complex than in our model

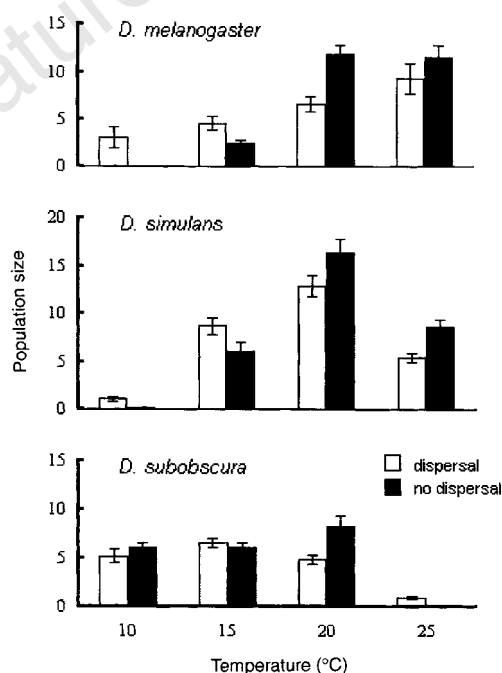


Figure 4 Comparison of *Drosophila* populations on their own, with and without dispersal. Error bars, ± 1 s.e. based on means of all measures.

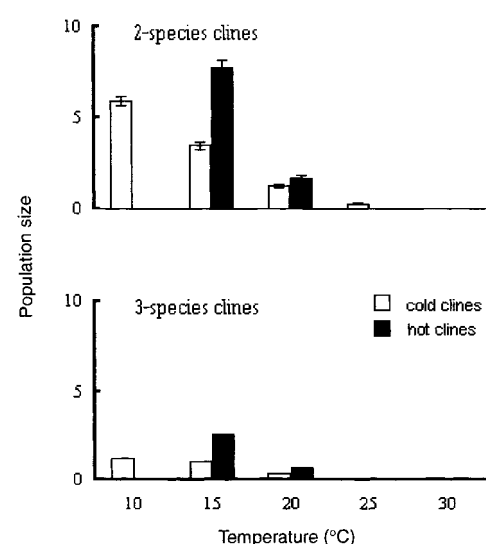


Figure 5 Comparison of *Drosophila subobscura* populations in cold and hot clines, either in two-species clines or three-species clines. Two-species clines include *D. simulans*; three-species clines also include *D. melanogaster*. The populations of *D. subobscura* at 15°C in hot clines are higher than in cold clines (the other species were unaffected). Error bars, ± 1 s.e. based on means of all measures.

system; hence, wherever dispersal and interactions operate in natural populations, we believe that global warming will provoke similar phenomena.

Our model system illustrates that horizontal (competition) and vertical (enemy–victim) interactions and dispersal markedly affect the distributions and abundances of species. Because these factors are omnipresent in nature, predicting changes in distribution and abundance under global warming by extrapolation of the climate envelope may lead to serious errors. Prediction using climate envelopes might be seen as a ‘null model’, but we predict that it will be frequently rejected given the accelerating changes initiated by global warming. Models incorporating dispersal and species interactions will be required for adequate predictions of the potentially serious applied consequences of global warming on conservation and medical and agricultural pest control. □

Methods

Cline construction and maintenance. The eight Perspex cages (each 100 × 150 × 300 mm) of a single cline were connected together, and to the pair in the neighbouring incubators, by tubing of 30 mm diameter which restricted adult dispersal between temperature zones to approximately 6% per day. Each cage held six tubes containing 50 ml standard cereal-based *Drosophila* medium which were replaced sequentially with fresh tubes. The reprovisioning frequency increased with temperature so the *Drosophila* at the four different temperatures received the same amount of food per generation. Honey was provided as food for adult *L. bouhardi* parasitoids. Nine single-species clines, three replicates for each species, were initiated by adding 25 male and 25 female flies to all eight cages of a cline, and three two-species clines were started similarly by adding 50 *D. simulans* and 50 *D. subobscura* to every cage. Five three-species clines were started with 50 flies of each of the three species in every cage, and four-species clines were started by adding wasps to five additional, four-week-old, three-species clines at the rate of 10% of the pre-existing *Drosophila* adults in each cage irrespective of species. Closed clines were created from established two-species clines by blocking the tubes between incubators with porous foam bungs, and hot clines were created by raising by 5 °C the temperatures of existing cold clines.

Estimating populations. The populations of adult *Drosophila* were assessed by weekly standardized partial counts of each cage. These counts and the real number of adult flies, irrespective of species, are closely related (no. of adults = $320 + (9.88 \times \text{count})$; $r = 0.814$, $P < 0.001$). Because *D. melanogaster* and *D. simulans* cannot be distinguished without detailed examination, the standard counts were adjusted by the proportions of these two species present in weekly samples of 100–150 flies withdrawn from each cage. To avoid the initial phases of population increase and to standardize the time period considered for each treatment, the analyses here are confined to counts made between weeks 5 and 25.

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Correspondence and requests for materials should be addressed to A.J.D. (e-mail: a.j.davis@leeds.ac.uk).

Automatic alerting does not speed late motoric processes in a reaction-time task

Steven A. Hackley* & Fernando Valle-Inclán†

* Department of Psychology, 210 McAlester Hall, University of Missouri, Columbia, Missouri 65211, USA

† Department of Psychology, Elviña Campus, University of La Coruña, 15071 La Coruña, Spain

When an irrelevant ‘accessory’ stimulus is presented at about the same time as the imperative signal in a choice reaction time-task, the latency of the voluntary response is markedly reduced¹. The most prominent cognitive theories agree that this effect is attributable to a brief surge in arousal (‘automatic alerting’), but they disagree over whether the facilitation is localized to a late, low-level motoric process² or to an earlier stage, the process of orienting to and then perceptually categorizing the reaction stimulus^{3,4}. To test these alternative hypotheses, we used the onset of the lateralized readiness potential (a movement-related brain potential) as a temporal landmark to partition mean reaction time into two time segments. The first segment included the time required to perceive the visual stimulus and decide which hand to react with; the second included only motoric processes. Presentation of an irrelevant acoustic stimulus shortened the first interval but had no effect on the second. We therefore rejected the motoric hypothesis.

The evidence that led Sanders² to propose a late motoric locus for the speeding of reaction time by automatic alerting was based on Sternberg’s⁵ additive factors method. These studies showed that the effects of task-irrelevant accessory stimuli do not interact with the effects of variables assumed to influence perceptual or decision processes, but they do interact with effects involving low-level motoric processes, such as tonic muscle tension. The additive factors method involves many assumptions that are difficult to verify, but the motoric hypothesis has also been supported by neurophysiological research. These experiments showed that the motoric processes underlying certain reflexes can be facilitated by accessory stimulation^{6,7}. However, more recent research⁸ indicates that the pattern of facilitation is quite different for voluntary and

reflexive reactions, so the relevance of these reflex studies to the understanding of alerting effects on reaction time is uncertain.

To test the competing hypotheses more directly, we measured the timing of motor cortex potentials with respect to both stimuli and responses in a choice reaction-time task. The component of interest was the lateralized readiness potential (LRP), an index of hand-specific response preparation⁹. To isolate this component from other event-related potentials, the waveforms recorded at the scalp site over motor cortex on the same side as the responding hand were subtracted from the waveforms obtained at the corresponding contralateral site. This difference function (the LRP) averages zero until the subject decides which hand to react with and then initiates hand-specific motor preparation. For waveforms computed with signal averaging synchronized to the visual stimuli, we measured the time interval extending from stimulus onset until LRP onset (or more precisely, the point at which 50% of peak amplitude was first reached). Then, using signal-averaged waveforms that were time-locked to response onset, we separately measured the time interval extending from LRP onset (again, the 50% point) until movement onset¹⁰.

The predictions are straightforward. If alerting speeds a perceptual or decision-level process, as hypothesized by Posner³, then the

stimulus-to-LRP interval should be shortened. As shown in the hypothetical waveforms at the bottom left portion of Fig. 1, this should be manifested as a displacement to the left of the stimulus-locked LRP on trials for which an accessory stimulus accompanies the reaction stimulus. Under this hypothesis, there should be no effect on the response-locked LRPs (bottom right, Fig. 1). If instead alerting facilitates a late low-level motoric process, as hypothesized by Sanders², then the interval extending from onset of the response-locked LRP until movement onset should be shortened by accessory stimulation (top right, Fig. 1). For the stimulus-locked LRP, the predictions are less clear. If, as suggested by the reflex data^{6,7}, the facilitation takes place downstream from the LRP, then the waveforms obtained on accessory and control trials should superimpose more or less precisely. If, on the other hand, the facilitation involves those cortical processes that mediate the LRP, it is still the case that there should be no effect on the absolute onset latency of the stimulus-locked LRP. However, a steeper slope should be observed on accessory trials as motor cortex activity more rapidly approaches the response threshold (top left, Fig. 1).

The LRPs were recorded in 20 undergraduates who had given their informed, written consent to participate. In each trial, the subjects were presented with a single letter 'S' or 'T', either 1.0° or 0.8° in size, at the centre of the computer screen. The difficult size discrimination determined whether the subjects should give a speeded, multifinger keypress response (Go trials, $P = 0.80$) or should withhold the reaction (NoGo trials, $P = 0.20$). The easily discriminated difference in shape (S versus T) indicated whether the left or right hand should be used. In half of the trials, an accessory stimulus (80 dB, 60 ms ramp, 150 ms long, tone pip) accompanied the visual reaction stimulus at a lead time of 30 ms.

In agreement with extensive behavioural research^{1-4,8}, reaction time was faster on accessory trials than on control trials (Go trial means = 524 and 544 ms, respectively; $F_{1,19} = 47.3$, $P < 0.0001$). Analyses of the LRPs indicated that the reaction-time effect was localized entirely within the stimulus-to-LRP time interval (Fig. 2, left; $t_{19} = 1.84$, $P < 0.05$, at the conventional 50% amplitude point; the effect was much stronger when measured at other time points). The response-locked LRPs for the accessory and control trials overlapped almost perfectly (Fig. 2, right; $t_{19} = 0.76$, n.s.). Also in agreement with predictions based on Posner's hypothesis³ (compare with Fig. 1), the rise time of the LRP did not differ between conditions for either the stimulus- or response-locked waveforms (measured between the 20% and 60% amplitude points; $t_{19} = 0$ and 0.22, for the S- and R-locked LRPs, respectively, n.s.).

In NoGo trials, a brief LRP was observed in the absence of electromyogram (EMG) activity (Fig. 2, left). This finding replicates previous results¹¹ and suggests that subjects began preparing the selected hand as soon as the shape of the letter had been discriminated. Then, when letter-size information was transmitted from the perceptual system, subjects aborted the planned response. The NoGo LRP was speeded by accessory stimulation even though

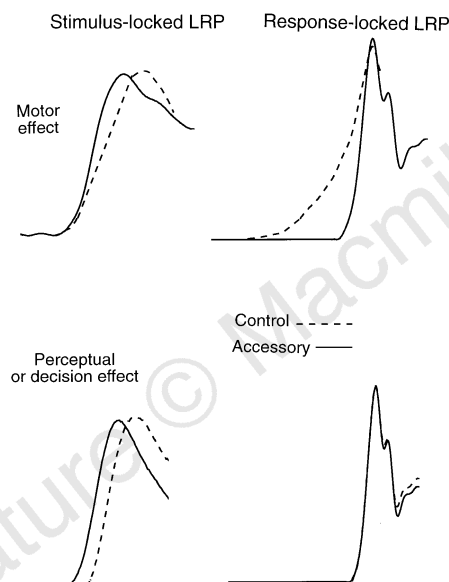
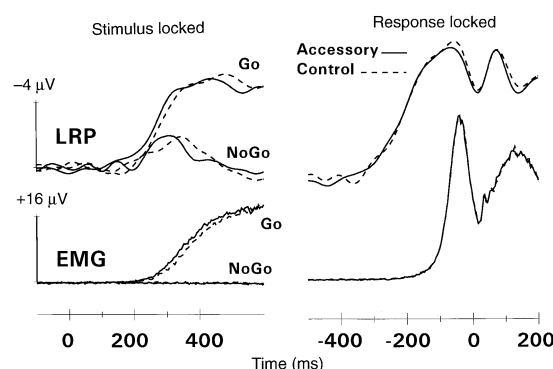


Figure 1 Predictions regarding the pattern of latency effects that should be observed under the two alternative hypotheses (upper and lower halves of the figure). The hypothetical waveforms shown at the right are time-locked to the initial keypress, which would occur just after the peak of the lateralized readiness potential (LRP), whereas those shown at the left are time-locked to the onset of the visual stimulus, which would take place at the beginning of the curves.

Figure 2 Grand average lateralized readiness potentials (LRPs) and forearm electromyograms (EMG) for trials in which the visual reaction stimulus was presented alone (dashed lines) or was accompanied by an irrelevant auditory stimulus that was presumed to increase momentarily the subject's level of arousal (solid lines). For the stimulus-locked waveforms shown on the left, the origin of the abscissa indicates onset of the visual stimulus and data for both Go and NoGo trials are shown. For the response-locked averages on the right, zero indicates onset of the keypress response on Go trials.



late, low-level motor processes were presumably never engaged ($t_{19} = 2.08$, $P < 0.05$, with measurement at 60% of peak amplitude; not significant at the 50% time point). This result (along with the other main findings) has been confirmed in follow-up studies.

The significant effect for NoGo LRP latency can exclude an alternative interpretation of alerting effects in choice reaction-time tasks (see ref. 12). Suppose that in a portion of the accessory trials subjects simply reacted to the onset of the tone pip without taking the time to analyse the visual stimulus. Because the tone pip conveyed no information about the required response, subjects would presumably have guessed wrongly for about half of the trials. As overt errors, these trials would have been excluded from further analysis. But trials with correct guesses would have been included in the analyses and would have produced artefactually shortened reaction times for accessory as compared to control trials.

The electrophysiological data contradict this hypothetical model. The polarity and brevity of the LRP on NoGo trials indicate that letter shape and size were correctly perceived and correctly mapped to responses in the vast majority of trials. This finding indicates that alerting effects were not produced by fast guess trials in which the decision process was bypassed. Re-analysis of the Go trial data also supported this conclusion. After correcting the LRP waveforms and the reaction-time distributions^{13,14} for the contaminating influence of fast guess responses, the size and reliability of the accessory stimulus effects remained virtually unchanged. In conjunction with the low error rate on accessory trials (1.9%), which did not differ from that on control trials (1.6%, $F_{1,19} = 1.76$, n.s.), these data indicate that subjects rarely if ever responded to the tone.

Having excluded fast guess reactions and the facilitation of late, low-level motor processes as explanations for automatic alerting effects, it must be concluded that some process that was completed by the time of LRP initiation was speeded by accessory stimulation. This facilitated process presumably would have been either visual feature analysis¹⁵, stimulus categorization³ or response selection¹⁶. Psychophysiological methods, along with others from the more general field of cognitive neuroscience are well suited for testing these hypotheses. □

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Correspondence and requests for materials should be addressed to S.A.H. (e-mail: shackley@showme.missouri.edu).

A small population of anterior cells patterns the forebrain during zebrafish gastrulation

Corinne Houart^{*†}, Monte Westerfield[†] & Stephen W. Wilson^{*}

^{*} Developmental Biology Research Centre, The Randall Institute, Kings College London, 26–29 Drury Lane, London WC2 5RL, UK

[†] Institute of Neuroscience, University of Oregon, Eugene, Oregon 97403, USA

^{*} Present address: Department of Anatomy and Development Biology, University College London, Gower Street, London WC1E 6BT, UK

During gastrulation in vertebrates, dorsal ectoderm is induced to form neural tissue that later gives rise to the brain and spinal cord. This induction depends on signals arising from a group of cells on the dorsal side of the gastrula. This group of cells constitutes the organizer^{1,2}. It is thought that the organizer initially induces neural tissue with anterior, or forebrain, character, and that other signals subsequently posteriorize neural tissue in the trunk^{2,3}. Here we show that development of the anterior central nervous system of the zebrafish embryo also depends on a small group of ectodermal cells located in the prospective head region. Removal of these ectodermal cells during gastrulation perturbs subsequent neural patterning and results in widespread cell death. Transplantation of these cells shows that they can induce forebrain-specific gene expression in more posterior regions of the neural plate. Our results indicate that an early step in neural patterning is the establishment of a small population of signalling cells within the most anterior region of the embryo. These cells are required for patterning and survival of the anterior brain.

The anterior ectoderm is thought to be a passive player in early neural development, responding to neural-inducing and patterning signals that come either through the plane of the ectoderm or from underlying mesendodermal cells². To test this theory, we ablated small populations of anterior ectodermal cells in the zebrafish embryo (Fig. 1a). By mid-gastrula stage in wild-type embryos, spatially restricted domains of ectoderm cells express early neural and non-neural markers^{4,5}, indicating that neural induction has begun. At this stage, a border is visible within the ectoderm, roughly at the interface between prospective neural and non-neural ectoderm (Fig. 1b). We ablated rows of ectodermal cells on the posterior side of this border, defining row 1 as the line of cells just posterior to the border (Fig. 1b), row 2 as a line of cells just posterior to row 1, and so on (Fig. 1a). Using this definition, we ablated each of the first ten rows of cells, each ablation removing a line of cells approximately spanning the width of the prospective neural plate (Fig. 1a). Most ablations produced no obvious consequences upon later development (Table 1). However, when either the first row or the sixth/seventh row was ablated, subsequent brain development was impaired (Table 1). These results suggest that regulatory interactions compensate for the loss of most rows of cells in the anterior ectoderm, but that row-1 and row-6/7 cells are non-replacable by mid-gastrulation stage. Preliminary analysis indicates that ablations of row 6/7 result in abnormalities in the mid-diencephalon (data not shown). We describe here the role of the most anterior row of cells, and show that this row is required for patterning the anterior forebrain.

Anterior mesendodermal derivatives of the organizer, usually referred to as the prechordal plate, are involved in patterning the head region¹. We therefore ascertained whether the abnormal brain development seen after row-1 ablation could be due to inadvertent damage to the prechordal plate. Sections of embryos in which row-1 cells had been labelled by intracellular injection of biotinylated

dextran showed that the leading edge of the involuting prechordal plate was posterior to row 1 at the time of ablation (Fig. 1c and d). Analysis of expression of *Fkd2*, *gooseoid* and *hgg1*, all of which mark the prechordal plate^{6,7}, confirmed this conclusion (Fig. 1c and d and data not shown). Furthermore, sections of embryos fixed immediately after row-1 ablation showed that the experimental procedure did not directly damage the prechordal plate (Fig. 1e).

In addition to the ectoderm and the prechordal plate, two other tissues could be affected by the ablation. These are the yolk syncytial layer (YSL), which expresses *Fkd2* and directly underlies the ectoderm (Fig. 1c), and the enveloping layer (EVL) of large flattened cells of the extra-embryonic periderm (Fig. 1c). Ablation of row 1 caused a bulging of the YSL into the ectodermal layer of the embryo, but the yolk cell membrane appeared undamaged as compared

with the membrane of embryos in which the ablation procedure deliberately violated the yolk cell (compare Fig. 1e with Fig. 1f). Finally, the ablation procedure probably damaged a few EVL cells although it seems unlikely that this damage could be the cause of subsequent central nervous system (CNS) defects as ablation of row-2/3 cells probably also damaged the same EVL cells without leading to CNS defects. These results suggest that it is the absence of the row-1 ectodermal cells that perturbs subsequent brain development.

To understand the phenotypic consequences of removal of the nine to twelve cells that constitute row 1, brain development was examined for alterations in morphology, cell death, gene expression and neuronal differentiation. Morphogenesis of the anterior brain is reasonably normal in row-1 ablated embryos: optic primordia

Figure 1 Ablation of row 1 removes ectodermal cells but does not affect the prechordal plate. All panels show mid-gastrula-stage embryos. **a**, Schematic drawing showing the approximate positions of each row of cells ablated in this study. Rows are indicated by numbers within the representations of individual ectodermal cells. **b**, Animal-pole view of a living embryo showing row-1 cells (arrowhead) on the posterior side of a border (arrows) between cells in the ectoderm. **c**, Sagittal section stained with toluidine blue. Row-1 cells (arrowhead in inset) were injected with biotinylated dextran, and mesendodermal and YSL nuclei (dark blue) were labelled with an anti-*Fkd2* antibody. Row-1 is underlain by *Fkd2*-positive nuclei in the YSL whereas cells of the prechordal plate mesendoderm are posterior to row-1. **d-f**, Sagittal sections of embryos in which row-1 cells were labelled with dextran (**d**, black arrowhead) or were ablated (**e**, black arrowhead), or in which the ablation needle deliberately penetrated the yolk cell (**f**, y represents yolk). The anterior limit of *gooseoid* expression in the prechordal plate (white arrowheads) is posterior to row-1. evl, enveloping layer cells; pc, prechordal-plate mesendoderm; ysl, yolk syncytial layer. The scale bar in **c** represents 100 μ m and other scale bars represent 25 μ m.

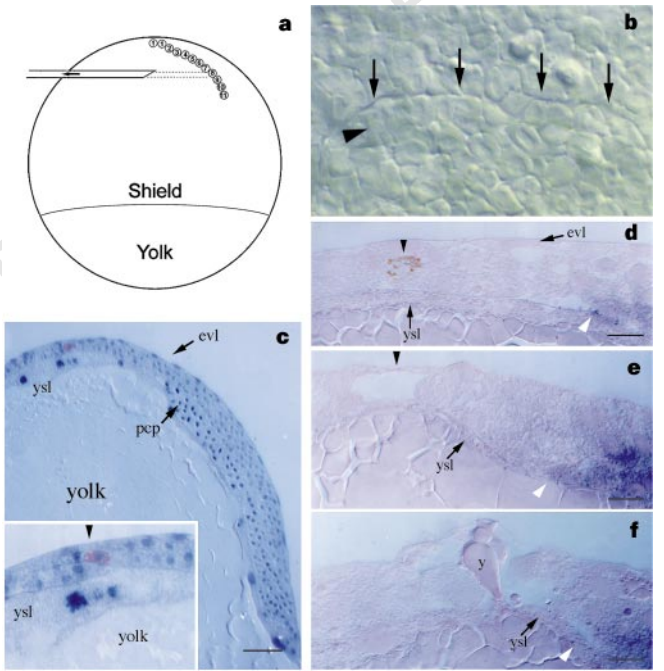


Figure 2 Ablation of row-1 cells at mid-gastrulation induces apoptosis in the brain. Lateral views of dissected whole brains in which terminal-transferase labelling has been used to detect cells (blue) undergoing programmed cell death in control (**a**, **c**) and row-1-ablated (**b**, **d**) at embryos at prim-5 (**a**, **b**) and prim-18 (**c**, **d**) stages. d, diencephalon; mb, midbrain; t, telencephalon. Scale bars in **a**, **b** represent 60 μ m and in **c**, **d** represent 75 μ m.

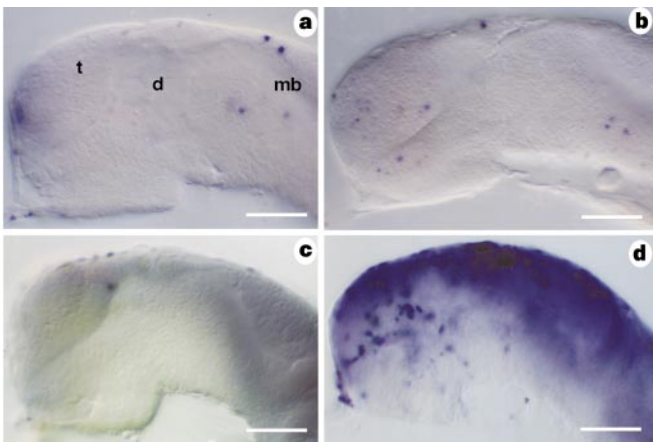


Table 1 Ablation of row-1 and row-6/7 affects brain patterning

Row number	1	2	3	4	5	6	7	8	9	10
Total no. of embryos	221	39	37	31	35	72	59	28	31	26
No. showing detectable brain defect	208	0	0	0	0	53	11	0	0	0
No. of dead embryos	13	2	4	2	3	5	3	1	3	3
Percentage showing brain defect	94	0	0	0	0	79	18	0	0	0

Row-1 cells and row-6/7 cells are specified and non-replaceable by mid-gastrula stage. The phenotype obtained after ablation of row-6/7 is distinct from that resulting after row-1 ablation. These results were obtained from analysis of ~400 embryos that were examined for brain defects either as living embryos or following labelling with anti-acetylated-tubulin antibody.

evaginate, and the forebrain divides into telencephalic and diencephalic territories. At the onset of the pharyngula stage, there is little apoptotic cell death in the brains of either control embryos (see Methods $n = 31$; Fig. 2a) or row-1 ablated embryos ($n = 37$; Fig. 2b). However, the brains of row-1-ablated embryos subsequently undergo pronounced cell death ($n = 45$; Fig. 2d), whereas control embryos show no comparable levels of apoptosis ($n = 34$; Fig. 2c).

To determine whether forebrain patterning is altered before apoptosis occurs, we examined the expression of two genes in the anterior/dorsal forebrain, *emx1* (ref. 8) and *dlx2* (ref. 9), and one gene, *shh* (ref. 10) that is expressed in more posterior and ventral forebrain tissue. At the onset of the pharyngula stage, expression of both *emx1* ($n = 19$) and *dlx2* ($n = 51$) was reduced or absent in row-1-ablated embryos compared with control embryos ($n = 24$ and 56 respectively; Fig. 3a–d). In contrast, *shh* was ectopically expressed in the anterior forebrain of row-1-ablated embryos ($n = 41$, Fig. 3e and f). These results indicate that telencephalic gene expression is lost and diencephalic gene expression expands before apoptosis occurs in the brains of row-1-ablated embryos.

Many early neurons and axons are positioned along boundaries of gene expression domains within the neuro-epithelium^{11,12}. As ablation of row-1 cells disrupts gene expression domains, we examined whether neuronal differentiation was also perturbed. In row-1-ablated embryos, differentiated neurons in the forebrain were reduced or absent at the start of the pharyngula stage ($n = 59$; Fig. 3h). In contrast, ablation of rows 2–5 had no obvious effect on neuronal differentiation ($n = 43$; Fig. 3g). Furthermore, expression of *Zash-1a* (ref. 13), a gene associated with neurogenesis, was drastically reduced in the forebrain of row-1-ablated embryos ($n = 32$; Fig. 3j).

Row-1 cells could be required for forebrain patterning either because their descendants normally contribute to all the forebrain territories affected by the ablation or because row 1 is involved in patterning adjacent neural tissue. Fate-mapping studies ($n = 49$) showed that row 1 contributes to the anterior telencephalon, anterior pituitary and nasal placode (Fig. 4a–c), whereas ablation of row 1 has phenotypic consequences throughout the anterior forebrain. To test whether row-1 cells can pattern adjacent regions of the neural plate, we examined whether transplantation of row-1 cells to more posterior regions (at the level of rows 7–8) of the prospective neural plate induced gene expression characteristic of the anterior forebrain (Fig. 4d). Following transplantation of row-1 cells at mid-gastrula stage, we observed ectopic *dlx2* expression (in 39 of 45 embryos) and *emx1* expression (in 20 of 25 embryos) in clusters of host cells surrounding or near the grafted cells (Fig. 4f and h). In contrast, transplantation of more posterior rows of cells had no obvious effects on subsequent patterns of *dlx2* or *emx1* expression ($n = 34$; Fig. 4e and g). These results indicate that row-1 cells and/or their descendants produce a signal that can induce expression of genes characteristic of anterior forebrain in cells that would normally form posterior diencephalon or midbrain. However, these observations do not reveal the stage at which row-1 cells have signalling activity.

Two sets of experiments addressed the question of whether row-1 cells show signalling activity during gastrulation. First, row-1 cells were injected with biotinylated fluorescent dextran at the mid-gastrula stage; these cells were then immediately ablated ($n = 8$) or left to develop to late-gastrula stage (90% epiboly; $n = 9$), at which time they were ablated. Embryos were subsequently fixed at bud or at ten-somite stages. Expression of *emx1* was absent in bud- and ten-somite-stage embryos in which row 1 had been ablated at mid-gastrulation (Fig. 5d–f). In contrast, embryos in which row-1 cells and their descendants were ablated at late gastrulation showed few or no alterations in subsequent expression of *emx1* (Fig. 5g–i). These results indicate that row-1 cells produce a signal required for induction of *emx1* during gastrulation, and that they need to

produce this signal only for a short period between mid- and late-gastrula stages. In support of these conclusions, transplantation of row-1 cells to more posterior locations in the neural plate at mid-gastrulation induces ectopic expression of *emx1* by bud stage ($n = 9$; Fig. 5c).

Our data indicate a new and previously unsuspected role for anterior ectodermal cells in the initial patterning of the anterior CNS. An early response to neural induction may be the establishment of a small group of anterior ectodermal cells that are required for subsequent patterning of the anterior brain. At later stages of development, fibroblast growth factor 8 (FGF8), secreted by the anterior neural ridge may influence gene expression in the telencephalon¹⁴. However, FGF8 is expressed too late^{14,15} for it to

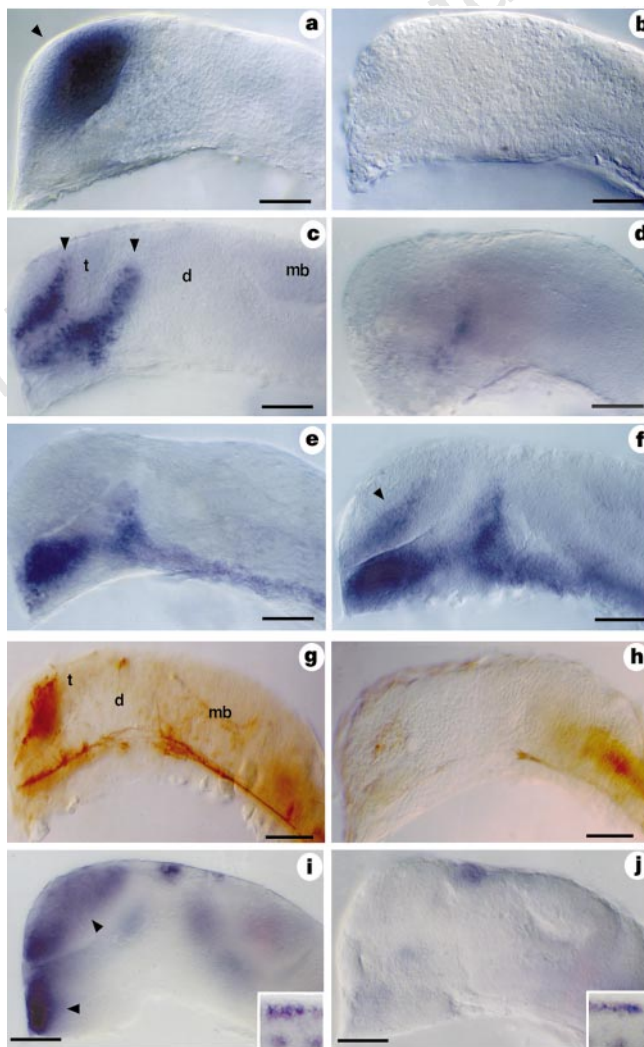


Figure 3 Ablation of row 1 disturbs gene expression patterns in the anterior forebrain and inhibits neurogenesis. All panels show lateral views of dissected brains, with anterior to the left. **a, b**, Expression of *emx1* (arrowhead in **a**), present in the telencephalon of a control embryo (**a**), is absent in the row-1-ablated embryo (**b**). **c, d**, expression of *dlx2*, present in the telencephalon and anterior diencephalon of a control embryo (**c**, arrowheads), is reduced in the row-1-ablated embryo (**d**). **e, f**, Expression of *shh* in control (**e**) and row-1-ablated (**f**) embryos. In the row-1-ablated embryo, telencephalic cells ectopically express *shh* (arrowhead). **g, h**, Neuronal differentiation in control (**g**) and row-1-ablated (**h**) embryos. In the row-1-ablated embryo, differentiated neurons are mostly absent from the forebrain. **i, j**, Expression of *zash-1a* (arrowheads) is present in the rostral forebrain of a control embryo (**i**) and nearly absent from this region in the row-1-ablated embryo (**j**). Expression in the spinal cord is similar in control and row-1-ablated embryos (insets). d, diencephalon; mb, midbrain; t, telencephalon. Scale bars represent 60 μ m.

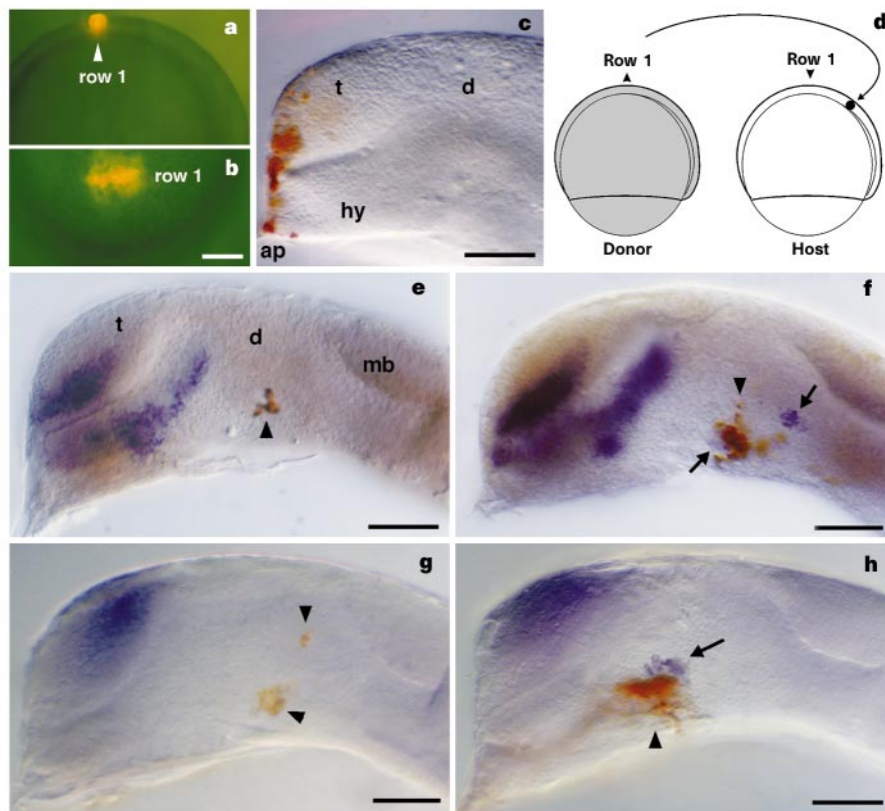


Figure 4 Row-1 cells have signalling activity. **a, b**, Lateral (**a**) and dorsal (**b**) views of a mid-gastrula embryo in which dil (orange) has been applied to row-1 cells. **c**, Lateral view of the brain of a pharyngula-stage embryo labelled as in **a**. Descendants of the row-1 cells (brown) are present in the telencephalon, anterior pituitary and olfactory placode (out of focus). **d**, Schematic drawing of a lateral view of mid-gastrula embryos, illustrating the transplantation. **e-h**, Embryos into which row-3 cells (**e** and **g**, arrowheads) or row-1 cells (**f** and **h**, arrowheads) had been transplanted at mid-gastrula stage. *dlx2* and *emx1* are ectopically expressed (arrows in **f** and **h**, respectively) near the grafted row-1 cells. ap, anterior pituitary; d, diencephalon; hy, hypothalamus; mb, midbrain; t, telencephalon. Scale bars in **a, b** represent 100 μm; other scale bars represent 60 μm.

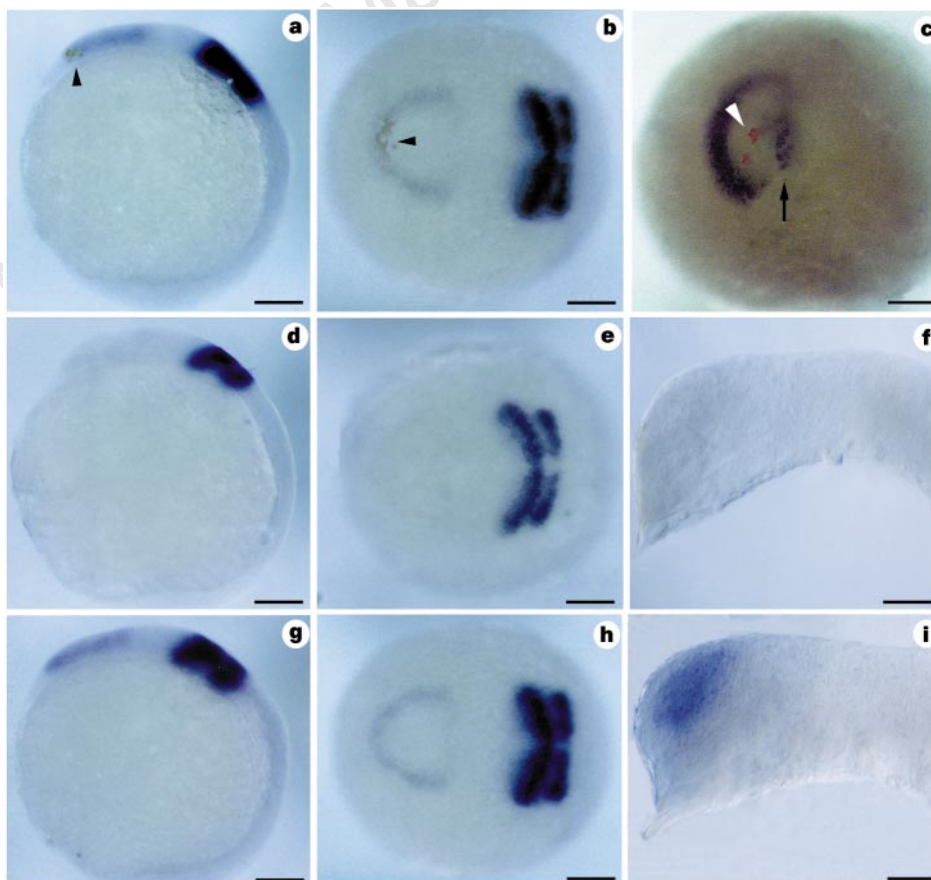


Figure 5 Row-1 cells signal during gastrulation. **a, b**, Lateral (**a**) and dorsal (**b**) views of bud-stage control embryos. *emx1* is expressed at the rostral margin of the neural plate and *krox20* is expressed in rhombomeres 3 and 5. Arrowheads indicate row-1 cells and their descendants. **c**, Bud-stage embryo in which *emx1* (arrow) is ectopically induced following transplantation of row-1 cells (brown, white arrowhead) to more caudal regions of the neural plate. **d-i**, Embryos in which row-1 cells were labelled at mid-gastrulation stage and were immediately ablated (**d-f**) or were ablated at late gastrula stage (**g-i**). Expression of *emx1* is absent at both bud (**d, e**) and ten-somite (**f**) stages following row-1 ablation at mid-gastrulation but is unaffected at these stages following ablation at late gastrulation (**g-i**). Scale bars in **f, i** represent 50 μm; other scale bars represent 100 μm.

be the row-1 signalling activity during gastrulation. Although our data concur with the possibility that anterior ectodermal cells acquire their inducing properties in response to signals from the organizer, this is not certain. Signals from endoderm in anterior regions of mouse and frog embryos are required for anterior development^{16–19}, and could be involved in induction of row-1 activity. It is unknown at present which cells in zebrafish correspond to the extra-embryonic endoderm of mice or deep endodermal cells of *Xenopus*. However, the YSL shows localized inductive activity²⁰, indicating that it could be a source of signals at the anterior end of the zebrafish embryo. The possible involvement of anterior endoderm in head patterning, together with the data we present here, indicate that early patterning of anterior regions of the vertebrate embryo may crucially depend upon cellular interactions occurring in anterior regions of the embryo. □

Methods

Zebrafish embryos were collected and staged as described²¹. We use the term mid-gastrula to denote the 70%–75% epiboly stage. To ablate ectodermal cells, embryos were mounted in 3% methyl cellulose in embryo medium²¹ and viewed on a fixed-stage Nikon Optiphot microscope; cells were removed by suction using a glass micropipette mounted on a hydrolic micromanipulator and attached to a 10 µl Hamilton syringe. For control ablations, cells from rows 2–5 were ablated or the cells removed from row 1 were placed back in their original positions. For fate-mapping, cells were labelled with dil dissolved in 100% ethanol or were individually injected iontophoretically with a mixture of fluorescent and biotinylated dextrans. For transplantations, donor embryos were injected with a mixture of fluorescent and biotinylated dextrans, and cells transplanted into host embryos were identified using a Vectastain kit to reveal biotin²². All subsequent antibody and *in situ* hybridization protocols followed standard procedures¹¹. For sectioning, embryos were embedded in methacrylate JB4 resin and cut at 7–10 µm on a Leica Ultracut microtome. *hgg1* was isolated by K. A. Barth in a random screen for developmentally expressed complementary DNAs.

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Correspondence and requests for materials should be addressed to C.H. (e-mail: vcgacor@ucl.ac.uk).

Staufen-dependent localization of *prospero* mRNA contributes to neuroblast daughter-cell fate

Julie Broadus*, Sal Fuerstenberg* & Chris Q. Doe

Department of Cell & Structural Biology, Howard Hughes Medical Institute, University of Illinois, Urbana, Illinois 61801, USA

The generation of cellular diversity is essential in embryogenesis, especially in the central nervous system. During neurogenesis, cell interactions or asymmetric protein localization during mitosis can generate daughter cells with different fates^{1–4}. Here we describe the asymmetric localization of a messenger RNA and an RNA-binding protein that creates molecular and developmental differences between *Drosophila* neural precursors (neuroblasts) and their daughter cells, ganglion mother cells (GMCs). The *prospero* (*pros*) mRNA and the RNA-binding protein Staufen (Stau) are asymmetrically localized in mitotic neuroblasts and are specifically partitioned into the GMC, as is Pros protein^{5–7}. Stau is required for localization of *pros* RNA but not of Pros protein. Loss of localization of Stau or of *pros* RNA alters GMC development, but only in embryos with reduced levels of Pros protein, suggesting that *pros* RNA and Pros protein act redundantly to specify GMC fate. We also find that GMCs do not transcribe the *pros* gene, showing that inheritance of *pros* RNA and/or Pros protein from the neuroblast is essential for GMC specification.

The *Drosophila* central nervous system (CNS) develops from stem-cell-like precursors called neuroblasts. Smaller daughter cells (GMCs) 'bud off' from the basal side of the neuroblasts; most GMCs then produce two postmitotic neurons. An important regulator of GMC development is the homeodomain protein Pros. Pros localizes to the apical cytoplasm of neuroblasts at late interphase⁷; at mitosis, it is translocated to the basal cortex where it forms a membrane-associated crescent, and it is subsequently inherited by the GMC in which Pros translocates into the nucleus^{5–7}. Asymmetric cortical localization of Pros is believed to keep Pros protein out of the neuroblast nucleus, and to rapidly generate nuclear Pros protein in the newborn GMC, where Pros establishes GMC-specific gene expression^{8,9}.

Here we describe the cell-cycle-specific asymmetric localization of *pros* mRNA in neuroblasts, which results in its selective partitioning into the daughter GMC. During interphase, most *pros* RNA is localized to the apical side of the neuroblast, where it is found either in the cytoplasm or associated with the cortex (Fig. 1a, Table 1). During mitosis, *pros* RNA is asymmetrically localized to the opposite side of the neuroblast in a basal cortical crescent (Fig. 1a; Table 1). At cytokinesis, *pros* RNA is specifically segregated into the GMC. Localization of *pros* RNA does not reflect a general

*These authors contributed equally to this work.

movement of poly(A⁺) RNA, as a different transcript (*seven-up*) is not localized at any phase of the neuroblast cell cycle (results not shown). Asymmetric cortical crescents of *pros* RNA can also be observed in mitotic sensory organ precursors and adult midgut precursors (results not shown).

The asymmetric localization of *pros* RNA suggests that one or more RNA-binding proteins may also be differentially segregated during neuroblast division. A good candidate for such a protein is the RNA-binding protein Stau, which is asymmetrically localized in the oocyte, is required for localization of *bicoid* and *oskar* RNA, and is detected in the embryonic CNS^{10,11}. We find that Stau shows cell-cycle-specific asymmetric localization in neuroblasts in a pattern that precisely matches the pattern of localization of *pros* RNA and Pros protein (Fig. 2). In late interphase, Stau is localized to the apical cytoplasm or cortex (Fig. 2a). From metaphase through to telophase, Stau is asymmetrically localized as a basal crescent, and segregates into the daughter GMC (Fig. 2b–e). A recent report¹² did not detect basal localization of Stau in neuroblasts, but we observed basal localization of Stau both *in vivo* and *in vitro*¹³, with two different antibodies, and this is consistent with a role for Stau in the basal localization of *pros* RNA (see below). Although Stau and Pros proteins are precisely co-localized in mitotic neuroblasts (Fig. 2f)¹³,

Stau protein localization is normal in *pros* null embryos (Fig. 2g), and Pros protein localization is normal in *stau* null embryos (Fig. 2h). Thus, Stau and Pros proteins are independently localized to the same region, probably by binding to a common anchoring protein.

To determine whether the localization of Stau and *pros* RNA in neuroblasts is microtubule-dependent, as is the localization of Stau and *bicoid* RNA in precellular embryos¹⁰, we depolymerized microtubules with colcemid, arresting neuroblasts in metaphase. Basal crescents of *pros* RNA (Fig. 1b) and Stau protein¹³ were easily observed, and the percentage of *pros* RNA crescents increased from 7% to 63% (*n* = 80) during colcemid treatment, indicating that new *pros* RNA crescents can form in the absence of microtubules. In contrast, actin microfilaments are absolutely required for anchoring Stau to the basal cortex¹³. Localization of Stau and RNA in neuroblasts could occur by diffusion and microfilament-based anchoring.

To determine whether Stau is required for localization of *pros* RNA in neuroblasts, we scored embryos (referred to as *stau* embryos) produced by homozygous *stau*^{D3} flies. These embryos lack both maternal and zygotic Stau. In mitotic neuroblasts of *stau* embryos, basal *pros* RNA localization is reduced from 71% to 27% (Table 1); apical RNA localization is reduced from 56% to 7% in

Table 1 Loss of *stau* reduces asymmetric localization of *pros* RNA

	Neuroblast cell-cycle phase	Subcellular distribution of <i>pros</i> RNA			
		Apical crescent	Basal crescent	Random crescent	Uniform cortical/cytoplasmic location
Wild type (<i>n</i> = 99)	Mitosis	0 (0%)	70 (71%)	1 (1%)	28 (28%)
<i>Stau</i> [−] (<i>n</i> = 91)	Mitosis	4 (4%)	25 (27%)	6 (7%)	56 (61%)
Wild type (<i>n</i> = 314)	Interphase	176 (56%)	1 (0%)	0 (0%)	137 (44%)
<i>Stau</i> [−] (<i>n</i> = 241)	Interphase	18 (7%)	6 (2%)	8 (3%)	209 (87%)

* Wild-type embryos are CyO/CyO or *stau*^{D3}/CyO embryos; *stau*[−] embryos are *stau*^{D3}/*stau*^{D3} embryos from *stau*^{D3}/*stau*^{D3} parents.

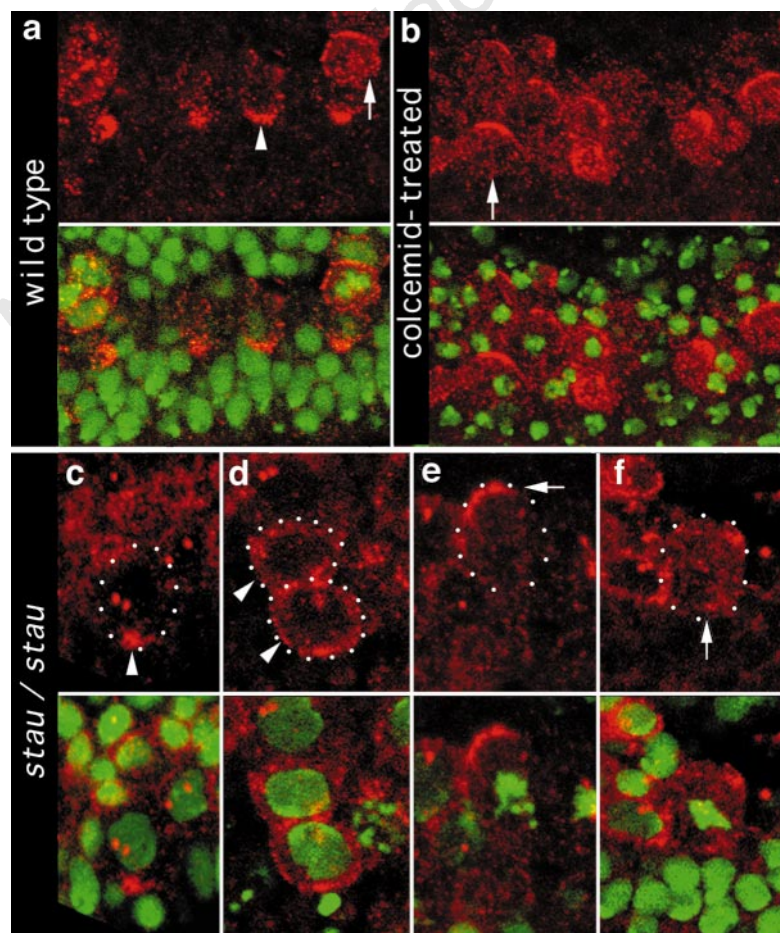


Figure 1 Asymmetric localization of *pros* RNA in neuroblasts requires Stau but not microtubules. Neuroblasts in stage 9–11 embryos were stained for *pros* RNA (red) and DNA (green). The bottom panels show results of double-labelling. Apical side is down; basal side is up. **a**, Wild-type embryo. Interphase neuroblasts have apical *pros* RNA (arrowhead); mitotic neuroblasts have basal cortical *pros* RNA (arrow). **b**, Colcemid-treated embryo. Neuroblasts lack microtubules yet show robust basal crescents of *pros* RNA (arrow). **c–f**, *stau* mutant embryos. **c**, **d**, Interphase neuroblasts with apical (**c**) or delocalized (**d**) *pros* RNA (arrowheads). **e**, **f**, Metaphase neuroblasts with basal (**e**) or delocalized (**f**) *pros* RNA (arrows).

interphase neuroblasts (Table 1). Most *stau*⁻ neuroblasts show uniform cytoplasmic or cortical *pros* RNA localization (Fig. 1). Similar results are seen in embryos lacking only zygotic *stau* expression (results not shown). It seems likely that apical Stau protein is required for apical *pros* RNA localization at interphase, and that basal Stau protein is required for basal *pros* RNA localization at metaphase. Crescents of *pros* RNA still form at low frequency in *stau* embryos (Fig. 1), however, suggesting that Stau is not the only factor that is able to localize *pros* RNA at the cortex. We conclude that the RNA-binding protein Stau is required for reliable apical and basal localization of *pros* RNA in neuroblasts.

What is the function of the asymmetric segregation of *pros* RNA

into GMCs? Hybridization with a *pros* intron probe shows that *pros* is transcribed in 67% of neuroblasts (*n* = 144; Fig. 3a), but is transcribed in only 2% of GMCs (*n* = 851; Fig. 3b). Thus, detection of *pros* RNA in GMCs^{7,9,14} (Fig. 3 insets) must reflect inheritance of *pros* RNA from the neuroblast. As GMCs clearly require *pros* function^{8,9}, but do not transcribe the *pros* gene, inheritance of *pros* RNA and/or Pros protein from the neuroblast is essential for GMC specification.

Loss of *pros* RNA localization—but not of Pros protein localization—in *stau* embryos does not alter GMC fate as judged by expression of the markers *even-skipped* (*eve*) or *fushi tarazu* (results not shown). To determine whether localized *pros* RNA and localized

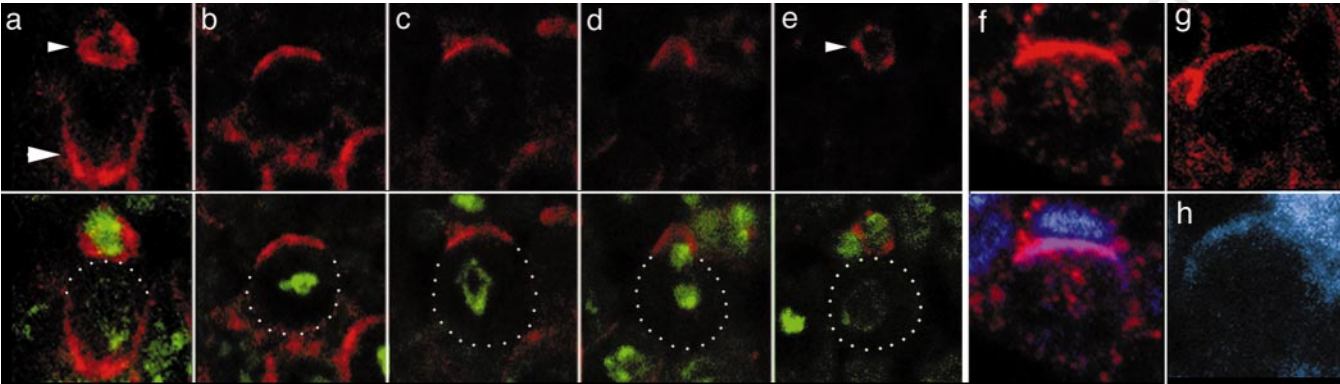


Figure 2 Asymmetric localization of Stau and Pros proteins in neuroblasts. **a–e**, Cell-cycle-specific localization of Stau protein (red) and *stau* DNA (green) in wild-type neuroblasts (bottom panels show results of double-labelling). Apical side is down; basal side is up. **a**, Interphase neuroblast with apically located Stau (large arrowhead; localization of Stau in the GMC cytoplasm is indicated by the small arrowhead). **b**, metaphase, **c**, anaphase and **d**, telophase neuroblasts with Stau

localized to the basal cortex. **e**, Stau is segregated into the GMC where it is cytoplasmic (arrowhead). **f**, Stau (red; top) and Pros (blue; bottom, merged with Stau image) proteins are co-localized at mitosis. **g**, Stau is localized normally in *pros*⁻ mitotic neuroblasts. **h**, Pros is localized normally in *stau*⁻ mitotic neuroblasts.

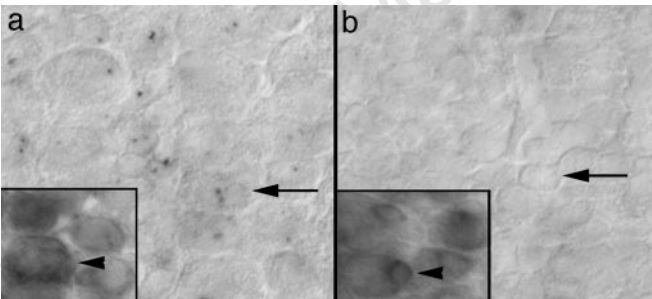


Figure 3 *pros* is transcribed in neuroblasts but not GMCs. A stage-10 embryo was labelled with a *pros* intron probe (**a**, **b**) or a *pros* complementary DNA probe (insets). **a**, Ventral view of the neuroblast layer. Most neuroblasts (arrow) show two chromosomal sites of *pros* transcription; not all sites of transcription are in focus. **b**, Ventral view of the adjacent GMC layer in the same embryo. GMCs (arrow) rarely show *pros* transcription; in this focal plane, none of the GMCs are transcribing *pros*. Insets: a *pros* cDNA probe shows high levels of *pros* RNA (arrowheads) in both neuroblasts and GMCs.

Table 2 Loss of Stau enhances a hypomorphic *pros* GMC phenotype

	Medial Eve ⁺ cells per embryo (<i>n</i>)		
	<i>pros</i> ^{c82}	<i>pros</i> ^{ss214}	<i>pros</i> ^{b149}
Stau ⁺	22.9 ± 4.7 (16)	26.2 ± 7.2 (16)	15.2 ± 6.5 (11)
Stau ⁻ (<i>stau</i> ^{D3})	10.1 ± 3.7 (7)	8.5 ± 4.6 (8)	6.3 ± 3.9 (6)
Stau ⁻ (<i>stau</i> ^{Y9})	6.3 ± 3.4 (3)	8.6 ± 2.4 (5)	n.a.

Segments A1–A7 were scored; the Eve⁺ phenotypes originate in GMCs (embryonic stage 10–11), but we scored neurons (stage 15–16) because the pattern is more reproducible. Medial Eve⁺ cells are defined as all Eve⁺ cells except for those cells of the lateral EL cluster²¹. Wild-type and homozygous *stau*^{D3} or *stau*^{Y9} embryos have ~112 medial Eve⁺ cells; *pros*-null embryos have ~2 medial Eve⁺ cells. n.a., not assayed.

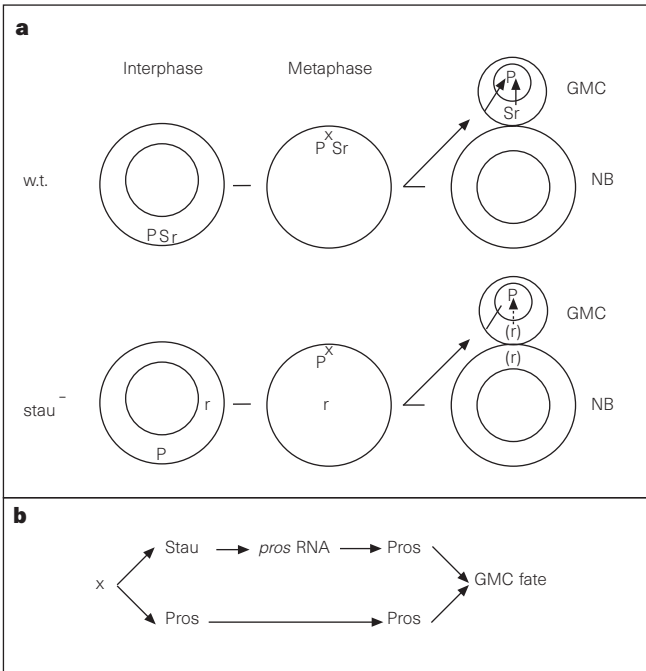


Figure 4 Localization and function of Stau and *pros* RNA in neuroblasts. **a**, Protein and RNA localization in wild-type and *stau* mutant neuroblasts. P, Pros protein; S, Stau protein; r, *pros* RNA; (r), lower level of *pros* RNA; X, putative Pros- and Stau-binding protein; NB, neuroblast. Apical, down; basal, up. **b**, Basal localization pathway: Stau/*pros* RNA and Pros protein are independently localized, perhaps by a common protein (X), and may act redundantly to provide levels of Pros that are necessary to establish GMC-specific gene expression.

Pros protein have redundant functions in GMC development, we used three hypomorphic (weakly active) alleles of *pros* (see Methods) to generate an 'intermediate' GMC phenotype: a reduction in medial Eve⁺ GMCs or their neuronal progeny. We then asked whether loss of *pros* RNA localization could enhance this phenotype. We found that hypomorphic *pros* embryos lacking Stau had less than half the number of medial Eve⁺ cells compared with hypomorphic *pros* embryos (Table 2). This enhancement is observed with two independent *stau* mutations in combination with three different *pros* alleles. These results indicate that *pros* RNA and Pros protein may act redundantly to establish GMC-specific patterns of gene expression (Fig. 4b). However, we cannot rule out the interesting possibility that delocalization of other RNA cargoes of Stau could contribute to the enhancement of the *pros* Eve phenotype.

Our results highlight a mechanism for reliably establishing neuroblast/GMC sibling fates, involving the coordinated but independent partitioning of *pros* RNA and protein into the GMC, where they may act redundantly to establish GMC-specific gene expression. Similarly, ASH1 RNA and Ash1p protein are co-localized and necessary to distinguish mother/daughter-cell fates in yeast^{15–17}. It remains to be seen whether the asymmetric co-localization of an mRNA and its protein product is a widely used mechanism for reliably establishing distinct daughter-cell fates.

Note added in proof: The newly discovered Miranda protein^{22,23} is likely to be protein X in Fig. 4. □

Methods

stau^{r9}, *stau*^{D3} and *pros*¹⁷ are null alleles^{8,11}, *pros*^{B149}, *pros*^{SS214}, and *pros*^{C82} produce decreased Pros-protein levels but normal Pros localization (C.-Y. Peng, J. B. Skeath and C.Q.D., observations). Permeabilized embryos at 4–5 h (ref. 18) were treated with 5 mg ml⁻¹ colcemid (Sigma) for 2 h at 25 °C. Antibody staining was performed using standard methods^{7,19} using mouse anti-Pros⁷, rabbit anti-Stau¹¹, and rat anti-Stau (S.F. and C.Q.D., results not shown). RNA *in situ* hybridization was performed as described previously²⁰; fluorescent detection was done with 1:200 fluorescein-isothiocyanate-conjugated anti-digoxigenin (Boehringer) with propidium iodide or sonicated phenylenediamine¹⁹ for DNA detection. Cell-cycle staging was based on DNA condensation and segregation. Detailed methods are available on request.

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Correspondence and requests for materials should be addressed to C.Q.D.

HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C

Veronique M. Braud^{*†}, David S. J. Allan^{*†}, Christopher A. O'Callaghan^{*}, Kalle Söderström[‡], Annalisa D'Andrea[‡], Graham S. Ogg^{*}, Sasha Lazetic[‡], Neil T. Young[§], John I. Bell^{*}, Joseph H. Phillips[‡], Lewis L. Lanier[‡] & Andrew J. McMichael^{*}

^{*} Institute of Molecular Medicine, John Radcliffe Hospital, Oxford OX3 9DS, UK

[‡] Department of Immunology, DNAX Research Institute of Molecular and Cellular Biology, Palo Alto, California 94304, USA

[§] Nuffield Department of Surgery, John Radcliffe Hospital, Oxford OX3 9DU, UK

[†] These authors contributed equally to this work.

The protein HLA-E is a non-classical major histocompatibility complex (MHC) molecule of limited sequence variability. Its expression on the cell surface is regulated by the binding of peptides derived from the signal sequence of some other MHC class I molecules^{1,2}. Here we report the identification of ligands for HLA-E. We constructed tetramers³ in which recombinant HLA-E and β 2-microglobulin were refolded with an MHC leader-sequence peptide, biotinylated, and conjugated to phycoerythrin-labelled Extravidin. This HLA-E tetramer bound to natural killer (NK) cells and a small subset of T cells from peripheral blood. On transfectants, the tetramer bound to the CD94/NKG2A, CD94/NKG2B and CD94/NKG2C NK cell receptors, but did not bind to the immunoglobulin family of NK cell receptors (KIR). Surface expression of HLA-E was enough to protect target cells from lysis by CD94/NKG2A⁺ NK-cell clones. A subset of HLA class I alleles has been shown to inhibit killing by CD94/NKG2A⁺ NK-cell clones^{4–6}. Only the HLA alleles that possess a leader peptide capable of upregulating HLA-E surface expression confer resistance to NK-cell-mediated lysis, implying that their action is mediated by HLA-E, the predominant ligand for the NK cell inhibitory receptor CD94/NKG2A.

The non-classical MHC class Ib molecule HLA-E has a broad tissue distribution⁷. Like its homologue, the mouse MHC class Ib Qa-1^{8–11}, HLA-E preferably binds to a peptide derived from amino-acid residues 3–11 of the signal sequences of most HLA-A, -B, -C, and -G molecules, but cannot bind its own leader peptide^{1,2}. Cell-surface expression of HLA-E depends on a functional transporter associated with antigen processing (TAP), and is upregulated by the binding of the peptides derived from MHC molecule signal sequences². The close correlation between the surface expression of HLA-E and of certain other MHC class I molecules suggested a possible role for HLA-E in NK-cell-mediated recognition of target cells.

To test this hypothesis, we constructed tetrameric complexes

containing HLA-E by refolding recombinant HLA-E and β 2-microglobulin (β 2m) molecules *in vitro* with a synthetic peptide (amino-acid sequence VMAPRTLVL) derived from residues 3–11 of the signal sequence shared by some HLA-B proteins. A biotinylation site was engineered in the carboxy terminus of the HLA-E heavy chain, allowing complexes containing HLA-E, β 2m and peptide to be enzymatically biotinylated and conjugated with phycoerythrin-labelled Extravidin to create tetrameric complexes. HLA-A and HLA-B tetrameric complexes bound specifically and efficiently to T-cell antigen receptors on antigen-specific CD8⁺ T cells from peripheral blood *in vitro*³.

We stained peripheral blood mononuclear cells (PBMC) from nine normal donors with the HLA-E tetramer and compared this staining with staining seen when using an HLA-A2 tetramer that was refolded with an Epstein–Barr virus (EBV) lytic cycle BMLF1 peptide epitope consisting of residues 259–267 (ref. 12). Lymphoid cells were stained with the HLA-E tetramer with high frequency (varying from 2% to 11% for different individuals) (Fig. 1a), whereas the HLA-A2 tetramer stained between 0% and 0.8% of the lymphocytes in EBV-seropositive donors (Fig. 1c). By setting an electronic gate on the lymphocytes that bind HLA-E tetramer, we saw that a large proportion of these cells were NK cells (CD3[−]CD56⁺); this proportion varied from 35% to 83%, mean 57%, between individuals. Also, T cells constituted a significant propor-

tion (range 12% to 60% CD3⁺ T cells; mean 34%; some of these T cells also expressed CD56) (Fig. 1b). Within the total CD3[−]CD56⁺ NK-cell population, 7% to 39% of cells (mean 18%) bound the HLA-E tetramer, and within the total CD3⁺ T-cell population, 0.7% to 3.2% of cells (mean 1.7%) bound the HLA-E-tetramer. In contrast, the HLA-A2 tetramer did not bind to CD56⁺ cells but, in EBV-seropositive donors, bound to EBV-specific CD3⁺CD8⁺ T cells (Fig. 1d), confirming the results of previous studies on the specificity of MHC-tetrameric complexes for T cells bearing specific T-cell antigen receptors³. In the donors tested, ~2% of the lymphocytes that bound HLA-E tetramer were CD4⁺ T cells, and typically 5% were CD19⁺ B cells. Similar staining was observed with the HLA-A2 tetramer and we are investigating whether this represents specific or nonspecific binding (data not shown).

Staining with HLA-E tetramer was abolished when the PBMC and the tetramer were incubated in the presence of the monoclonal antibody HP3D9 (ref. 13) against CD94, an NK cell receptor belonging to the C-type lectin superfamily¹⁴ (Fig. 2a). We confirmed the interaction between HLA-E and CD94 by staining a number of well-characterized CD94⁺ NK-cell clones with HLA-E tetramer and demonstrating that another anti-CD94 monoclonal antibody (DX22) (ref. 4) completely inhibited binding of the HLA-E tetramer (Fig. 2b, and data not shown). No staining of CD94⁺ NK-cell clones with HLA-A2 tetramer was found (data not shown).

Table 1 Inhibition of NK-cell-mediated killing of 721.221 cells by cell-surface expression of HLA-E bound to class I leader-sequence peptides

721.221 cells transfected with HLA class I alleles	Inhibition of killing by CD94/NKG2A ⁺ NK clones [*]	Presence of leader-sequence peptide capable of binding to HLA-E†	HLA leader sequence peptide (residues 3–11)	Concentration of peptide required to obtain 50% of binding to HLA-E‡
721.221	–	–§		
721.221-A*0201	+	+	VMAPRTLVL	0.06 μ M
721.221-A*0211	+	+	VMAPRTLVL	0.06 μ M
721.221-A*2501	+	+	VMAPRTLVL	0.06 μ M
721.221-A*2403	+	+	VMAPRTLVL	0.06 μ M
721.221-A*3601	+	+	VMAPRTLLL	0.05 μ M
721.221-B*0702	+	+	VMAPRTLVL	0.06 μ M
721.221-Cw*0102	+	+	VMAPRTLIL	0.3 μ M
721.221-Cw*0401	+	+	VMAPRTLIL	0.3 μ M
721.221-Cw*0304	+	+	VMAPRTLIL	0.3 μ M
721.221-Cw*0801	+	+	VMAPRTLIL	0.3 μ M
721.221-G	+	+	VMAPRTFLF	0.3 μ M
721.221-B*1501	–	–	VTAPRTLVL	>100 μ M
721.221-B*5101	–	–	VTAPRTLVL	>100 μ M
721.221-B*5801	–	–	VTAPRTLVL	>100 μ M
721.221-B*4601	–	–	VTAPRTLVL	>100 μ M
721.221-B*5401	–	–	VTAPRTLLL	>100 μ M
721.221-B*5501	–	–	VTAPRTLLL	>100 μ M

^{*} See ref. 4.
[†] The presence of a leader-sequence peptide capable of binding to HLA-E upregulates cell-surface expression of HLA-E, as measured on 721.221 and 721.221 cells transfected with HLA-A or -B alleles using the antibody DT9 which recognizes HLA-E and HLA-C alleles.
[‡] A peptide-binding assay was developed *in vitro*¹. Results are expressed as a ratio of optical densities referred to as percentage of binding to HLA-E.
[§] The HLA-A, -B, -C, and -G negative 721.221 cells express HLA-E and HLA-F, which have a shorter leader sequence and lack the appropriate peptide that is able to bind to HLA-E.

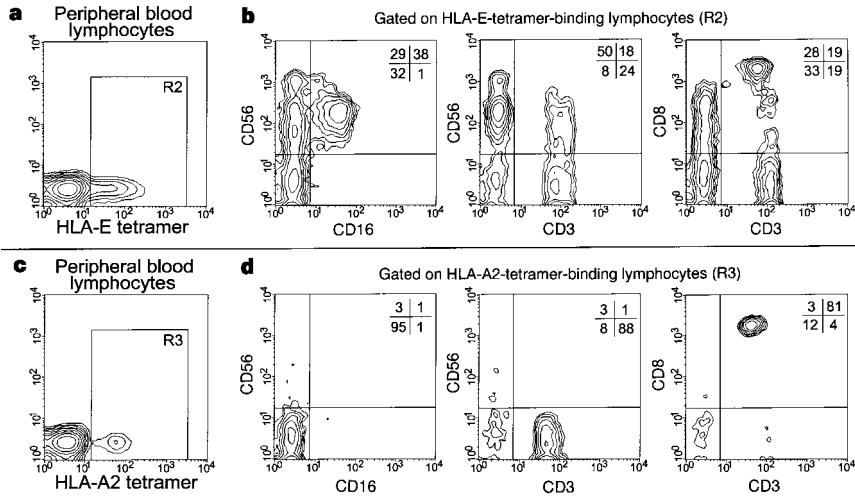


Figure 1 The HLA-E tetramer binds NK cells and a subset of T cells. Flow-cytometry analysis of gated peripheral blood lymphocytes from normal EBV-seropositive donor V.B. using (a) HLA-E tetramer/phycoerythrin refolded around the leader-sequence-peptide residues 3–11 from some HLA-B alleles or (c) HLA-A2 tetramer/phycoerythrin refolded around the EBV lytic cycle BMLF1 259–267 peptide epitope¹². The phenotypes of lymphocytes binding (b) HLA-E tetramer or (d) HLA-A2 tetramer were further investigated in triple colour stains as indicated. Percentages in each quadrant are listed in the upper right.

CD94 is expressed at the cell surface as a heterodimer with NKG2A, B (a splice variant of NKG2A), C, or E, members of the NKG2 family¹⁵⁻¹⁷. To characterize further the NK cell receptor that interacts with HLA-E, we stained 293T cells transfected with these receptors. We did not observe HLA-E tetramer staining on 293T cells transfected with CD94 or NKG2A alone (Fig. 3, and data not shown). The use of a strong promoter enabled some surface expression of NKG2A in the absence of CD94. In contrast, HLA-E tetramer did bind to 293T cells cotransfected with CD94 and NKG2A, CD94 and NKG2B, or CD94 and NKG2C (Fig. 3). We monitored the expression of these proteins using a polyclonal rabbit serum that reacts with the CD94/NKG2A, CD94/NKG2B and CD94/NKG2C heterodimers¹⁵. This result was confirmed using either mouse pre-B Ba/F3 cells that were stably transfected with CD94/NKG2C or NK-cell clones that expressed the inhibitory receptor CD94/NKG2A (Fig. 2b, and data not shown). Carbohydrates found on HLA-E are not necessary for binding, as the recombinant HLA-E used to make the tetramer was produced in *Escherichia coli*. This is surprising, given that both CD94 and NKG2 are members of the C-type lectin superfamily. Carbohydrate residues may form additional points of interaction, however, increasing the affinity of binding. Recombinant HLA-E produced in *E. coli* did not bind to and stain NK cells when used as single refolded HLA-E β 2m peptide molecules rather than as tetramers.

HLA-E does not interact with other killer-cell inhibitory receptors (KIR), as no staining with the HLA-E tetramer was seen on Ba/F3 cells transfected with KIR2DL1 (NKAT1 or p58), KIR2DL3 (NKAT2 or p58), KIR3DL1 (NKAT3 or p70), KIR3DL2 (NKAT4 or p70/140), KIR2DS2 (NKAT5 or p50), KIR2DL2 (NKAT6 or p58) or KIR2DS4 (NKAT8 or p50) (ref. 18). Furthermore, staining of

PBMC with HLA-E tetramer was not blocked by any of the following antibodies: EB6 (anti-KIR2DL1), GL183 (anti-KIR2DL3, anti-KIR2DS2 and anti-KIR2DL2), DX9 (anti-KIR3DL1), or 5.133 (anti-KIR3DL1 and anti-KIR3DL2) (data not shown). Thus, the CD94/NKG2 receptors seem to be specific receptors for HLA-E recognition.

NK cells expressing an inhibitory CD94/NKG2A receptor do not kill 721.221 cells transfected with certain HLA-A, -B, -C, or -G alleles, but are able to lyse these transfected cells in the presence of neutralizing antibodies against CD94 or MHC class I molecules^{4-6,19-21}. A marked correlation between the presence of an HLA class I leader-sequence peptide that is capable of binding to HLA-E, causing its surface expression, and the specificity of the CD94/NKG2A inhibitory receptor is seen (Table 1). All the MHC class I alleles that, upon transfection, protect 721.221 cells from killing by CD94/NKG2A⁺ NK-cell clones have a peptide that is capable of binding to the endogenous HLA-E. Similarly, all HLA alleles that are incapable of protecting 721.221 cells against these NK-cell clones lack an HLA-E-binding leader peptide. We confirmed, by immunoprecipitation, that the anti-class I antibody DX17, which inhibits interactions between class I molecules and CD94/NKG2A, also recognizes HLA-E.

To determine whether the presence of an HLA-E-binding leader peptide that induces surface expression of HLA-E is enough to provide protection against CD94/NKG2A⁺ NK-cell clones, a chimeric complementary DNA (GLS-B*5801) was generated. It contained the leader segment of HLA-G (from which a peptide can bind to HLA-E; Table 1) and the extracellular, transmembrane and cytoplasmic domains of HLA-B*5801 (an HLA molecule that is not implicated in recognition by CD94/NKG2A receptors⁴). Stable

Figure 2 Staining with the HLA-E tetramer is inhibited by anti-CD94 antibodies. **a**, Peripheral blood lymphocytes from normal donor S.R.J. were stained with the anti-CD94 antibody HP3D9 (ref. 13) (1 in 50 dilution of ascites) then with fluorescein isothiocyanate/anti-mouse IgG (Fab')₂, HLA-E tetramer/phycoerythrin alone, or HLA-E tetramer/phycoerythrin in the presence of HP3D9 (1 in 50 dilution), which inhibited staining with HLA-E tetramer. **b**, The NK-cell line NKL²⁷ expressing the NK cell receptor CD94/NKG2A but none of the KIR molecules was stained with the anti-CD94 antibody DX22 (ref. 4) (1 μ g) followed by phycoerythrin-labelled anti-mouse IgG, HLA-E tetramer/phycoerythrin alone or HLA-E tetramer/phycoerythrin in the presence of 1 μ g of DX22 antibody which inhibited staining with HLA-E tetramer. Percentages in each quadrant are listed in the upper right. The HLA-A2 tetramer refolded around the HTLV-I Tax peptide²³ did not bind to NKL cells (data not shown).

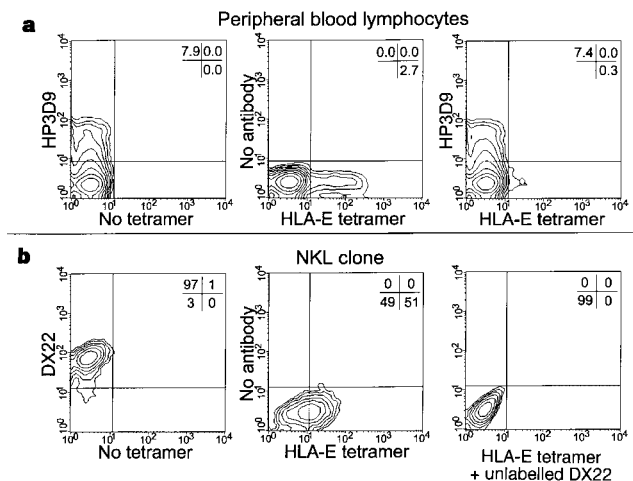
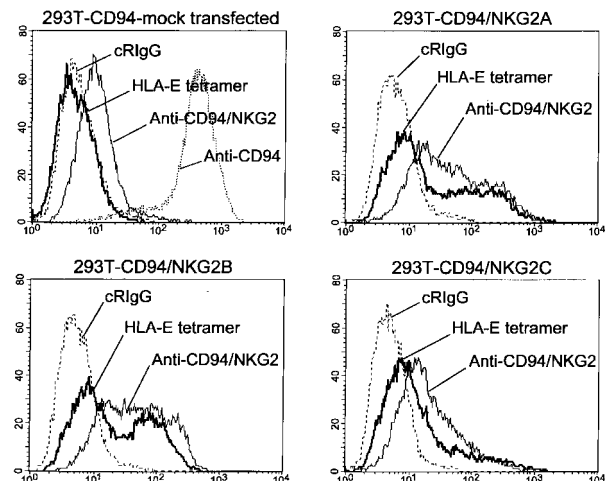
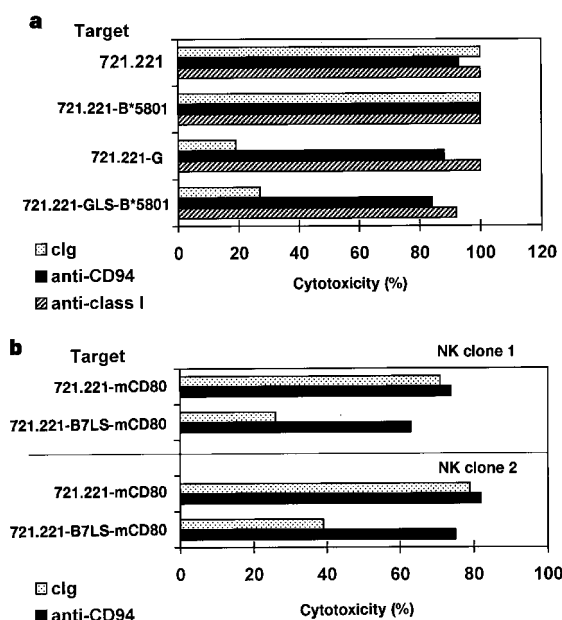


Figure 3 HLA-E binds to the NK cell receptors CD94/NKG2A, CD94/NKG2B and CD94/NKG2C. 293T cells stably transfected with CD94 were transiently transfected with NKG2A, NKG2B, NKG2C (ref. 15) or a control plasmid. Immunofluorescent staining was performed using anti-CD94 antibody DX22-phycoerythrin, rabbit pre-immune serum (cRlgG) (1 in 500 final dilution) or rabbit anti-CD94/NKG2-heterodimer serum (anti-CD94/NKG2) (1 in 500 final dilution) followed by fluorescein isothiocyanate/anti-rabbit IgG, or with HLA-E tetramer-phycoerythrin. No staining with the HLA-A2/HTLV-I Tax peptide tetramer was seen.





721.221 cell line transfectants were selected and analysed for susceptibility to lysis by NK-cell clones expressing CD94/NKG2A receptors. As shown in Fig. 4a, an NK-cell clone expressing a CD94/NKG2A receptor efficiently killed untransfected 721.221 cells as well as 721.221 cells transfected with wild-type HLA-B*5801. However, protection against NK-cell-mediated lysis was conferred by expression of the chimaeric GLS-B*5801 molecule but reversed in the presence of antibodies against either CD94 or HLA class I molecules. A chimaeric cDNA (B7LS-mCD80) containing the leader segment of HLA-B*0702 (with a peptide that can bind to HLA-E; Table 1) and the extracellular, transmembrane and cytoplasmic domains of mouse CD80 was transfected into 721.221 cells and tested for lysis by CD94/NKG2A⁺ NK-cell clones. There was less lysis of 721.221 cells expressing the B7LS-mCD80 molecule but not of cells expressing wild-type CD80, and protection was reversed by anti-CD94 but not control antibodies (Fig. 4b). These results indicate that an HLA-E-binding leader peptide alone is enough to protect 721.221 cells from lysis by NK-cell clones expressing inhibitory CD94/NKG2A-type receptors.

These results (Table 1, Figs 3 and 4) clearly identify HLA-E as a ligand for CD94/NKG2A. We are at present studying whether CD94/NKG2A interacts only with HLA-E or whether it also interacts with HLA-A, -B, -C or -G molecules. The latter possibility seems unlikely because an HLA-A2 tetramer refolded around an EBV peptide stained a different population of PBMC (Fig. 1d) and similar observations have been made with complexes of HLA-A2 or HLA-B8 with several other peptide epitopes^{3,22}. Furthermore, HLA-A2 refolded around a Tax-peptide epitope of human T-lymphotropic virus-I (HTLV-I) (ref. 23) did not bind to CD94/NKG2A transfectants or to NK cells expressing CD94/NKG2 receptors. Thus, HLA-A2 does not seem to bind to CD94/NKG2A but it might bind to CD94/NKG2A when complexed with certain, as yet unidentified, peptides. The human cytomegalovirus protein UL18 can also protect 721.221 cells from NK-cell-mediated lysis²⁴, possibly by involving CD94 receptors. We are investigating whether this can be explained by the binding of UL18 leader peptides to the endogenous HLA-E molecules in 721.221 cells.

The CD94/NKG2A and CD94/NKG2B complexes deliver inhibitory signals to NK cells, but HLA-E also binds to CD94/NKG2C which has been shown to activate cytolytic activity in NK-cell transfectants²⁵. Thus, HLA-E is involved in regulating NK-cell-mediated cytotoxicity both positively and negatively. Our results demonstrate a new role for a non-classical class I molecule, HLA-E,

Figure 4 HLA-E mediates inhibition of NK cells through interaction with CD94/NKG2A. **a**, Lysis of 721.221 cells expressing HLA-B*5801, HLA-G or a chimaeric molecule (GLS-B*5801) containing the HLA-G leader sequence and the extracellular, transmembrane, and cytoplasmic domains of HLA-B*5801 by a representative NK-cell clone expressing the CD94/NKG2A receptor. Assays were performed at an effector to target ratio of 0.5:1, in the presence of control immunoglobulin (clg), anti-CD94 (DX22), or anti-HLA class I (DX17) at 5 μ g ml⁻¹. **b**, Lysis of 721.221 cells expressing mouse CD80 or a chimaeric molecule (B7LS-mCD80) containing the HLA-B*0702 leader sequence and the extracellular, transmembrane, and cytoplasmic domains of mouse CD80 by two representative NK-cell clones expressing the CD94/NKG2A receptor. Assays were performed at an effector-to-target ratio of 1:1 in the presence of control immunoglobulin (clg) or anti-CD94 (DX22) at 10 μ g ml⁻¹.

and identify its predominant receptor on lymphocytes. It remains to be determined whether the strong preference of HLA-E for binding signal-sequence-derived peptides is needed simply to allow cell-surface expression of HLA-E, or whether it is implicit in recognition of HLA-E by CD94/NKG2 receptors. □

Methods

HLA-E was cloned by reverse transcription polymerase chain reaction (RT-PCR), with primers COO7 and COO6, from RNA extracted from monocytes of an HLA-E*0101 homozygous individual. The amino-terminal nucleotide sequence was synonymously altered by PCR mutagenesis using the primers CO17 and COO6 to optimize protein expression from the pGMT7 vector in *E. coli*. The coding sequence for the extracellular portion of HLA-E (residues 1–276) was amplified using the primers CO17 and CO23 and recloned into a pGMT7 derivative to produce the expression plasmid COO92, which contains the BirA recognition and biotinylation site in frame at the 3' end of the HLA-E heavy chain. Primers were as follows: COO6, 5'-GTGGGCTAAGCTTACGGCTTCCATCTCAGGGTGACGGGCTC-3'; COO7, 5'-CTACGGGCATATGGTAGATGGAACCTCCTTTTACTCTCC-3'; CO17, 5'-CCGTACCTCGAGCATATGGGTTCTCATTCTTTAAATATTTTCATACTTCTGTATCTAGACCCGGCCG-3'; and CO23, 5'-TGGTGTCTAGAGGATCC TGGCTTCCATCTCAGGGTGACGGGCTCG-3'.

HLA-E tetrameric complexes were generated nearly as described³. Briefly, HLA-E and β 2m proteins were overexpressed in *E. coli* strains BL21 (DE3) pLysS and XA90 respectively, purified from inclusion bodies, solubilized into a urea solution, then refolded by dilution *in vitro* with a synthetic peptide (VMAPRTVLL) shared by some HLA-B leader sequences (Research Genetics). Complexes of the HLA-E heavy chain, β 2m and leader-sequence peptide were biotinylated with BirA enzyme (provided by M. Davis), purified by Superdex gel filtration and Mono-Q ion-exchange chromatography (Pharmacia), then complexed in a 4:1 molar ratio with Extravidin-phycoerythrin (Sigma). 2.2 μ g HLA-E tetramer/phycoerythrin (488K) was used in each flow-cytometry stain of 10⁶ PBMC or transfectants.

Human NK-cell clones were established and cultured as described²⁶. Cytotoxicity assays were performed as described⁴. A chimaeric cDNA containing the leader segment of HLA-G and the extracellular, transmembrane, and cytoplasmic domains of HLA-B*5801 was generated by PCR using the following oligonucleotide primers: sense primer 1, 5'-GCGTCTAGAATGGTGGTCATGGCACCCCGA-3'; antisense primer 1, 5'-CATGGAGTGGGAGCCGGCCAGGTCTCGGT-3'; sense primer 2, 5'-GGCTCCCACTCCATGAGGTAT-3'; and antisense primer 2, 5'-AAGCTTCAAGCTGTGAGAGACA-3'.

PCR was performed using a wild-type HLA-G cDNA as a template with primer set 1 and using wild-type HLA-B*5801 cDNA as a template with primer

set 2. Products from these PCR reactions were mixed and used as templates for a subsequent reaction with sense primer 1 and antisense primer 2. The product was digested with *Xba*I and *Hind*III and ligated into the pBjneo vector.

A chimaeric cDNA containing the leader segment of HLA-B*0702 and the extracellular, transmembrane and cytoplasmic domains of mouse CD80 was generated by PCR using the following oligonucleotide primers: sense primer 3,5'-ACCGAGACCTGGGCGCTTGATGAACAACCTG-3'; antisense primer 3,5'-GCAAGCTTCTAAAGGAAGACGGTCTGTTC-3'; sense primer 4,5'-GGGCGTCGACCCGGACTCAGAATCTCTCAGACGCGGAG-3'; and antisense primer 4,5'-CAGTTGTTTCATCAACGGCCAGGTCTCGGT-3'.

PCR was performed using a wild-type mouse CD80 cDNA as a template with primer set 3 and using wild-type HLA-B*0702 cDNA as a template with primer set 4. Products from these PCR reactions were mixed and used as templates for a subsequent reaction with sense primer 4 and antisense primer 3. The product was digested with *Sal*I and *Hind*III and ligated into the pBjneo vector. PCR products were verified by sequencing. 721.221 B-lymphoblastoid cells were transfected with the wild-type and chimaeric cDNAs and selected as described²⁶.

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Correspondence and requests for materials should be addressed to V.M.B. (e-mail: vbraud@worf.molbiol.ox.ac.uk).

A B-cell-homing chemokine made in lymphoid follicles activates Burkitt's lymphoma receptor-1

Michael D. Gunn*, Vu N. Ngo†, K. Mark Ansel†, Eric H. Ekland†, Jason G. Cyster† & Lewis T. Williams*‡

* Cardiovascular Research Institute and †Department of Microbiology and Immunology, University of California at San Francisco, San Francisco, California 94143, USA

‡ Chiron Corporation, Emeryville, California 94608, USA

Secondary lymphoid organs (spleen, lymph nodes and Peyer's patches) are divided into compartments, such as B-cell zones (follicles) and T-cell zones, which provide specialized environments for specific steps of the immune response. Migration of lymphocyte subsets into these compartments is essential for normal immune function, yet the molecular cues guiding this cellular traffic are poorly defined. Chemokines constitute a family of chemotactic cytokines that have been shown to direct the migration of leukocytes during inflammation^{1,2} and which may be involved in the constitutive homing of lymphocytes into follicles and T-cell zones^{3–8}. Here we describe a novel chemokine, B-lymphocyte chemoattractant (BLC), that is strongly expressed in the follicles of Peyer's patches, the spleen and lymph nodes. BLC strongly attracts B lymphocytes while promoting migration of only small numbers of T cells and macrophages, and therefore is the first chemokine to be identified that is selective towards B cells. An orphan chemokine receptor, Burkitt's lymphoma receptor 1 (BLR-1), has been found to be required for B-cell migration into lymphoid follicles⁶. We show that BLC stimulates calcium influx into, and chemotaxis of, cells transfected with BLR-1. Our results indicate that BLC functions as a BLR-1 ligand and may guide B lymphocytes to follicles in secondary lymphoid organs.

To identify novel chemokines that might play a role in lymphocyte homing, we hybridized mouse tissues *in situ* with antisense transcripts of expressed sequence tags (ESTs) that showed homology to chemokines. One such EST (IMAGE Consortium Clone 596050) hybridized strongly to spleen, Peyer's patches and lymph nodes (Fig. 1d–f) but weakly or not at all to multiple non-lymphoid tissues (data not shown and Fig. 1l). We refer to this transcript and the protein it encodes as BLC. In the spleen, BLC hybridized to the B-cell-rich zones, or follicles, present in the outer region of the white pulp cords (Fig. 1d). A strong signal was detected in a reticular pattern within the follicle and at the outer boundary where the follicle meets the surrounding marginal zone (Fig. 1d, j). In Peyer's patches, expression of BLC was strongest within germinal centres, sites at which B cells undergo somatic mutation and affinity maturation⁹, and extended into the surrounding mantle zone (Fig. 1f). Expression in lymph nodes was again concentrated in a reticular pattern within the follicles, but expression was variable and was not seen in all follicles (Fig. 1e, k). Northern blotting revealed a 1.2-kilobase (kb) transcript in wild-type spleen, Peyer's patches and lymph nodes, but not in resting B or T cells (Fig. 1l). BLC expression was reduced by 85% in spleens of lymphocyte-deficient *RAG1*-knockout mice (Fig. 1l), suggesting that lymphocytes provide a stimulus that promotes BLC expression in non-lymphoid splenic cells. Accumulation of follicular dendritic cells (FDCs) in lymphoid tissues depends on the presence of B and T lymphocytes¹⁰. Furthermore, FDCs have extensive processes that extend throughout lymphoid follicles in a pattern that is similar to the BLC *in situ* hybridization pattern¹¹. These results indicate that FDCs may be a

source of this novel chemokine.

To identify the full-length complementary DNA encoding BLC, we searched for ESTs contiguous to the clone used for hybridization. Sequence analysis of four overlapping clones revealed a cDNA of 1,162 base pairs (bp), containing an open reading frame that encoded a putative protein of 109 amino acids with a predicted 21-amino-acid leader peptide (Fig. 2a). This sequence contained four cysteines in a pattern typical of the CXC family of chemokines¹. BLC has most similarity to the CXC chemokine GRO α (Fig. 2b). We also identified a cluster of six human EST clones encoding a protein with 64% amino-acid similarity to murine BLC. This protein is probably human BLC (Fig. 2b). A sequence-tagged site (STS) derived from this sequence (Genbank accession number G14456) has been mapped to chromosome segment 4q21 (ref. 12). This places the BLC gene near genes encoding most known CXC chemokines, including interleukin (IL)-8, GRO, IP-10, and PF4 (ref. 2). Interestingly, the protein with the greatest similarity to mouse and human BLC is Meq-sp, a product of the Marek's disease virus (MDV) Eco Q gene (Fig. 2b). MDV is a lymphotropic avian herpesvirus that causes a lethal disease in chickens. The disease is characterized by early cytolytic infection of B cells and subsequent development of T-cell lymphomas¹³. Meq-sp was identified in MDV-infected cells and was not previously recognized to contain a consensus chemokine motif¹⁴.

These results indicate that BLC may be a B-lymphocyte chemoattractant. To test this possibility, chemotaxis assays were performed with lymphocytes from mouse spleen, using baculovirus-expressed BLC estimated to be >95% pure by silver staining. BLC induced a strong chemotactic response in B cells in experiments using either total spleen lymphocytes (Fig. 3a) or purified B cells (Fig. 3b), showing that BLC acts directly on B cells. In contrast, BLC showed limited activity towards T cells carrying CD4 or CD8 antigens (Fig. 3c, d). The B-cell response was chemotactic rather than chemokinetic, as cells incubated with BLC in the absence of a BLC gradient did not migrate (Fig. 3g). Stromal-cell-derived factor-1 α (SDF-1 α), previously described as the most efficacious chemokine for resting lymphocytes¹⁵, attracted fewer B cells than

BLC and lacked any B-cell specificity (Fig. 3a–d), highlighting the unique properties of this chemokine and leading us to name it as we have. BLC had weak but reproducible chemotactic activity for spleen monocytes/macrophages (Fig. 3e) but, in contrast to many CXC chemokines, showed no chemotactic activity towards granulocytes (Fig. 3f). Despite its efficacy as a B-cell attractant (Fig. 3a, b), BLC had a potency less than that of most chemokines, possibly because the baculovirus-expressed protein is not fully active. An alternative possibility is that a chemokine that is expressed constitutively within lymphoid tissues does not need to be highly potent.

All chemokines studied thus far signal via pertussis-toxin-sensitive G-protein-coupled receptors² and this is also true for BLC, as pertussis-toxin-pretreated B cells failed to migrate (Fig. 3h). Recent studies of mice with a targeted disruption of the orphan chemokine receptor BLR-1 indicated that this receptor is required for B-cell homing to follicles in spleen and Peyer's patches⁶. We therefore tested whether BLC could signal through BLR-1. Human embryonic kidney 293 cells stably transfected with mouse BLR-1 showed a dose-dependent calcium flux in response to BLC (Fig. 4a), whereas several other chemokines did not stimulate such a response and did not desensitize these cells to BLC (Fig. 4b). BLC did not stimulate a calcium flux in 293 cells transfected with the chemokine receptors CCR1, CCR2 or CXCR2, showing that the response of BLR-1-transfected cells to BLC was specific (Fig. 4c, d and data not shown). Using BLR-1-transfected 300-19 pre-B cells, a more complete dose-response curve was obtained; this showed that the response to BLC is saturable (Fig. 4e). Non-transfected 300-19 cells did not express detectable BLR-1 (Fig. 4g) and failed to respond to BLC (Fig. 4e).

We next tested the ability of BLC to stimulate chemotaxis through BLR-1. Jurkat T cells transfected with BLR-1 showed a chemotactic response towards BLC, whereas BLR-1-negative cells failed to respond (Fig. 4f). The maximum migration of transfected Jurkat cells occurred at a lower BLC concentration than was necessary for maximum migration of B cells, despite substantially lower expression of BLR-1 on the Jurkat cells (Fig. 4g). This may be due to our use of an amino-terminal epitope-tagged receptor in these cells, or

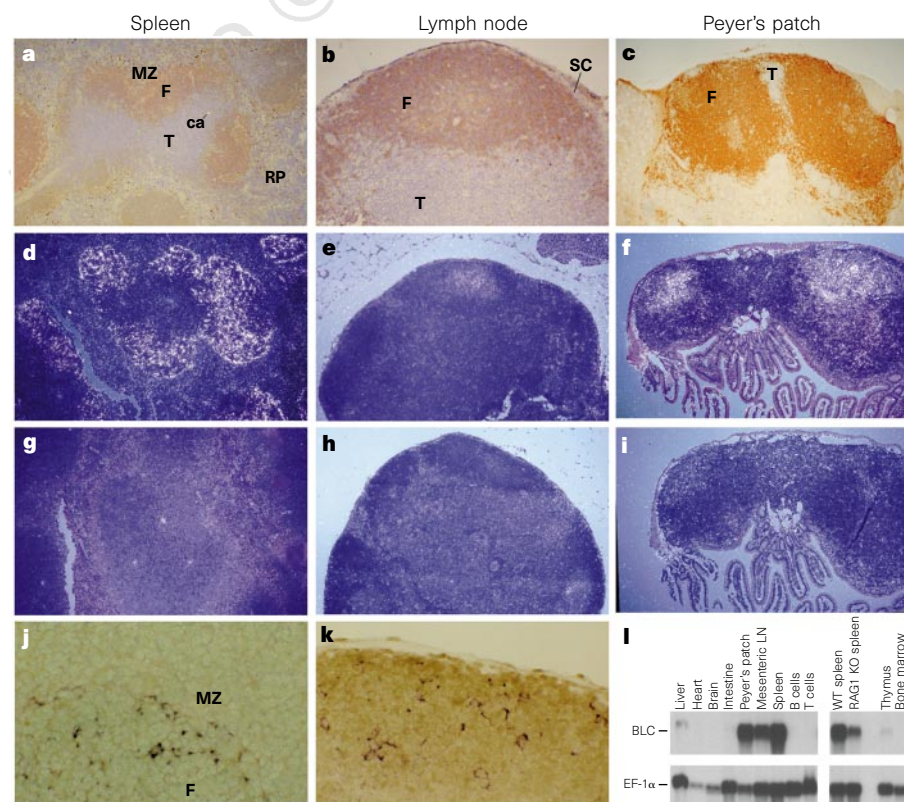


Figure 1 Expression pattern of BLC mRNA in mouse tissues. **a–c**, Identification of follicles by immunohistochemistry with anti-B220 is shown for orientation. Indicated structures are: ca, central arteriole; F, follicle; MZ, marginal zone; RP, red pulp; sc, subcapsular region; T, T-cell zone. **d–f**, Dark-field micrographs of hybridization of tissues with an antisense, ³⁵S-labelled BLC probe (2-week exposure). Signal is seen as white dots. **g–i**, Lack of hybridization to a ³⁵S-labelled sense BLC probe (8-week exposure). **j–k**, Bright-field micrographs showing hybridization with digoxigenin-labelled antisense BLC probe. Signal is seen as black staining. Original magnifications are $\times 10$ (**a**, **c**, **d–f**), $\times 20$ (**b**), and $\times 64$ (**j**, **k**). **l**, Northern-blot analysis showing levels of BLC mRNA in the indicated cells and tissues. EF-1 α hybridization indicates amount of total RNA loaded in each lane. KO, knockout; LN, lymph node; WT, wild type.

perhaps the coupling of receptors to downstream signalling pathways is different in mouse B cells and human Jurkat T cells. Mouse 300-19 pre-B cells transfected with wild-type BLR-1 showed a chemotactic response that had a similar dependence on the concentration of BLC to the response of purified B cells (data not shown). The chemotactic response of freshly isolated B cells, T cells, and Mac1⁺ cells to BLC (Fig. 3) correlates with the reported expression of BLR-1 in all B cells and in subsets of CD4⁺ and CD8⁺ T cells^{6,16,17} and with the expression of a variant form of BLR-1 in monocytes¹⁸. Our findings show that BLR-1 confers cells with responsiveness to BLC and that the responsiveness to BLC of cells from mouse lymphoid tissues correlates with the reported expression of BLR-1.

We have described a novel CXC chemokine, BLC, which is

expressed in the follicles of the spleen, Peyer's patches and lymph nodes and is a strong B-cell chemoattractant. BLC's expression pattern, chemotactic activity, and ability to stimulate cells expressing BLR-1 suggest that it is a physiological BLR-1 ligand, acting to direct the migration of B lymphocytes to follicles in secondary lymphoid organs. Although BLR-1 is required for B-cell migration into splenic and Peyer's patch follicles, it is not needed for B-cell localization in lymph-node follicles⁶. This, together with our inability to detect BLC expression in all lymph-node follicles, indicates that other B-cell-specific chemokines may exist in lymph nodes. It is also possible that there are other BLC receptors that may function in lymph nodes. MDV, which is transmitted by inhalation but rapidly infects B cells¹³, may have exploited the strong chemoattractant property of a BLC-like chemokine to attract target cells into

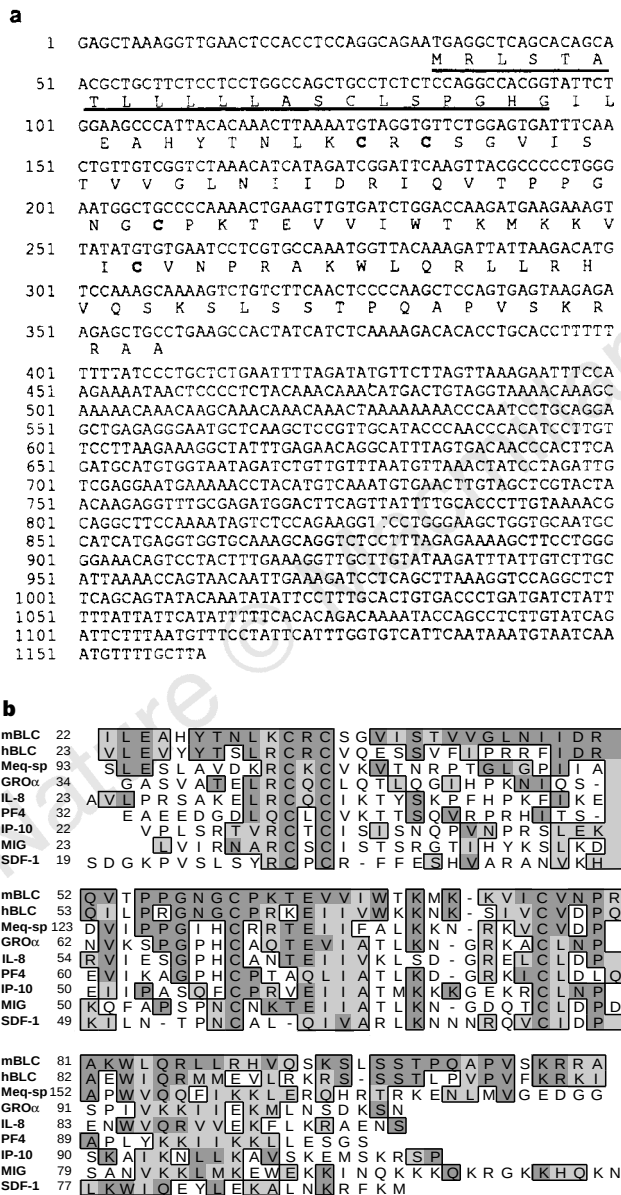


Figure 2 BLC sequence and alignment with other protein sequences. **a**, Nucleotide and deduced amino acid sequence of mouse BLC. The signal peptide, as determined by N-terminal sequencing, is underlined. Conserved cysteine residues are shown in bold. **b**, Alignment of the mouse (M) BLC protein sequence with those of human (h) BLC, GROα, IL-8, PF4, IP-10, MIG, SDF-1, and MDV-encoded Meq-sp. Identical amino acids are shaded dark grey. Conserved amino acids are shaded light grey. All shaded regions are boxed. Hyphens represent gaps inserted for optimum alignment. Numbers represent the position of the first amino acid shown in full-length protein.

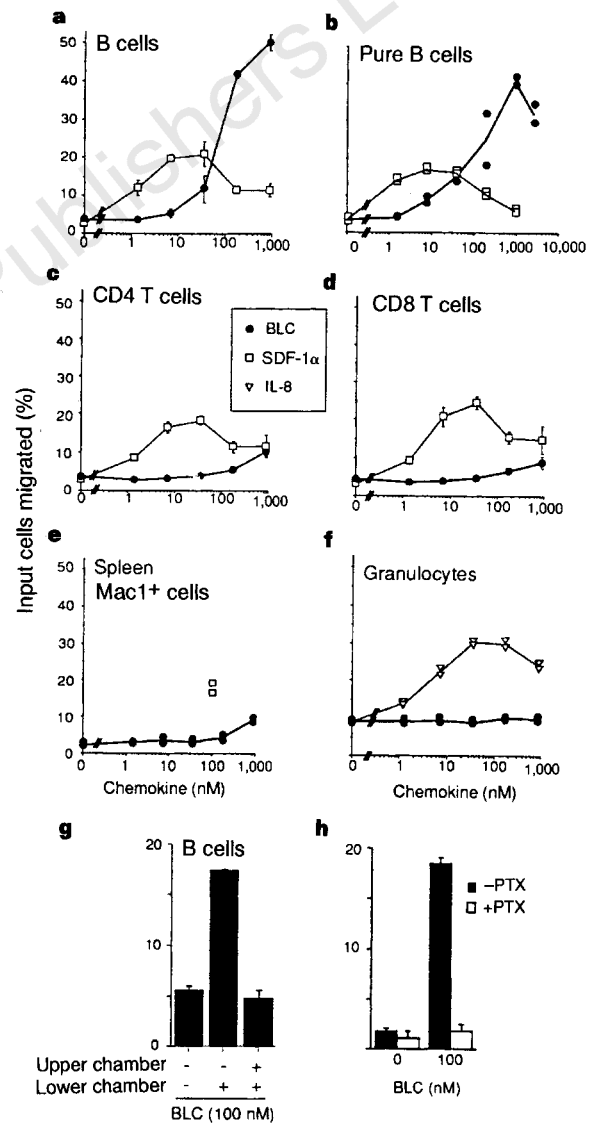


Figure 3 Chemotactic activity of BLC on leukocyte subtypes. Results are expressed as the percentage of input cells of each subtype migrating to the lower chamber of a transwell filter. Panels show migration of: **a**, B cells; **b**, purified B cells; **c**, CD4⁺ T cells; **d**, CD8⁺ T cells; **e**, monocytes/macrophages; **f**, granulocytes, towards BLC. Positive controls are SDF-1α (**a-e**) and IL-8 (**f**). **g**, Failure of B cells to migrate in the absence of a BLC gradient. BLC was added to the upper or lower chamber of the apparatus as indicated. **h**, Inhibition of BLC-induced migration by pretreatment of cells with pertussis toxin (PTX). Data points represent the mean $\pm$ s.d. for triplicates; individual data points are shown for duplicates. Each experiment was performed a minimum of two times.

the lung for infection. Our results and the identification of chemokines, expressed in lymphoid T-cell zones, that strongly attract resting T cells^{19–21} indicate that chemokines may be the major cues promoting cell compartmentalization within lymphoid tissues. □

Methods

Sequence analysis. Pattern searches of the NCBI EST database using TFASTA²², with human monocyte chemoattractant protein-1 as a template, retrieved human and mouse ESTs for BLC. BLAST²³ searches with these sequences identified contiguous ESTs. IMAGE Consortium [LLNL] cDNA clones 596050, 598232, 617961, and 749241 (ref. 24) were obtained from Genome Systems Inc. (St Louis, MO) as *EcoRI*–*NotI* inserts in the pT73-Pac vector, and sequenced. Similarity scores were calculated using the Blossum 30 matrix.

RNA expression studies. For northern-blot analysis, messenger RNA from mouse tissues or purified cells was subjected to gel electrophoresis, transferred to Hybond-N⁺ membranes (Amersham), and probed using randomly primed mouse BLC EST 596050, which spans bases 10–532 of the BLC cDNA. For

in situ hybridizations, paraffin sections (5 µm) from C57BL/6 mice were deparaffinized, fixed in 4% paraformaldehyde, and treated with proteinase K. After washing in 0.5 × SSC, the sections were covered with hybridization solution, prehybridized for 1–3 h at 55 °C, and hybridized overnight with sense or antisense ³⁵S-labelled riboprobe transcribed from the mouse BLC EST 596050. After hybridization, sections were washed at high stringency, dehydrated, dipped in photographic emulsion NTB₂ (Kodak), stored at 4 °C for 2–8 weeks, developed, and counterstained with haematoxylin and eosin. In some experiments, frozen sections were hybridized with sense or antisense digoxigenin-labelled riboprobes, immunostained with alkaline phosphatase coupled anti-digoxigenin antibody, and developed with NBT/BCIP as described (http://www.cco.caltech.edu/~mercer/htmls/Big_In_Situ.html). Immunohistochemistry with anti-B220 antibody was as described³.

Production of recombinant proteins. The mouse BLC EST 596050 was cloned into the pVL1393 baculovirus transfer vector and cotransfected with BaculoGold (Pharmingen) into SF9 cells according to the manufacturer's instructions. For protein production, SF21 cells were infected at a multiplicity of infection of 10–20 and cultured in serum-free media for 60 h. Conditioned media was cleared, loaded onto a HiTrap heparin-affinity column (Pharmacia), and eluted with a 0.2–1 M NaCl gradient in 50 mM HEPES (pH 7.9). Fractions containing BLC were pooled, run on a C-18 reverse phase HPLC column (Vydac), and eluted with an acetonitrile gradient. SDS-PAGE and silver staining of this preparation showed a single protein band of the expected molecular mass for BLC (10K) that represented >95% of the total protein. Protein concentration was measured using the Bio-Rad protein assay. Protein-sequence analysis identified the isoleucine at position 22 as the amino terminus of the mature recombinant protein.

Chemotaxis. Lymphocytes and macrophages were obtained from spleens of C57BL/6 mice. For macrophage chemotaxis, B cells were depleted by passage over a MACS column (Milteny Biotec) after incubation with biotinylated anti-B220 antibodies and streptavidin-coated magnetic beads. Granulocytes were obtained from mouse bone-marrow suspensions. Mouse BLR-1-transfected Jurkat cells were obtained by transfection with pREP4 (Invitrogen) containing the mouse BLR-1-coding region¹⁷, isolated by reverse transcription with polymerase chain reaction from mouse spleen RNA, and an N-terminal prolactin leader sequence and FLAG epitope²⁵ (provided by S. Coughlin). Positive clones were identified using anti-FLAG antibody M1 (Kodak). Chemotaxis assays were performed as described¹⁵ using 10⁶ cells per transwell and subsets of migrating cells were identified by flow cytometry using antibodies specific for B220, CD4 and CD8 (Pharmingen) and Mac-1 (Caltag, South San Francisco, CA). In some experiments, B cells were purified to >94% by MACS depletion of CD43⁺ cells. Granulocytes were identified by their characteristic large side-scatter profile. The absolute number of each cell type added to transwells was: 5.2 × 10⁵ B cells; 9.5 × 10⁵ purified B cells; 1.7 × 10⁵ CD4 T cells; 1.3 × 10⁵ CD8 T cells; 3.8 × 10⁵ granulocytes and 1.1 × 10⁵ Mac1⁺ spleen cells. In some experiments, cells were preincubated with 100 ng ml⁻¹ pertussis toxin (List Biology Labs, Campbell, CA) for 2 h at 37 °C. IL-8 (R&D Systems) and synthetic human SDF-1α (N33A) synthesized by native chemical ligation (a gift from M. Siani) were used as positive controls. SDF-1α (N33A) has identical activity to native human and mouse SDF-1α (refs 26, 27).

Flow cytometry and calcium fluorimetry of transfected 293, Jurkat, and 300-19 cells. Native mouse BLR-1 was subcloned into plasmid vector pBK-CMV (Stratagene) and used to transfect HEK 293 cells and 300-19 pre-B cells (provided by T. DeFranco). G418-resistant clones were tested for BLR-1 expression using an affinity-purified rabbit antiserum that is specific for the mouse BLR-1 N terminus (C. Timmons, C. Hsu and J.G.C., unpublished observations) and fluorescein isothiocyanate anti-rabbit secondary antibody (Caltag). HEK293 cells expressing CCR1, CCR2 and CXCR2 were provided by I. Charo. Calcium-mobilization studies were performed as described²⁸ using a Hitachi 4500 spectrometer. Intracellular calcium concentrations were calculated using the Hitachi 4500 intracellular cation measurement program.

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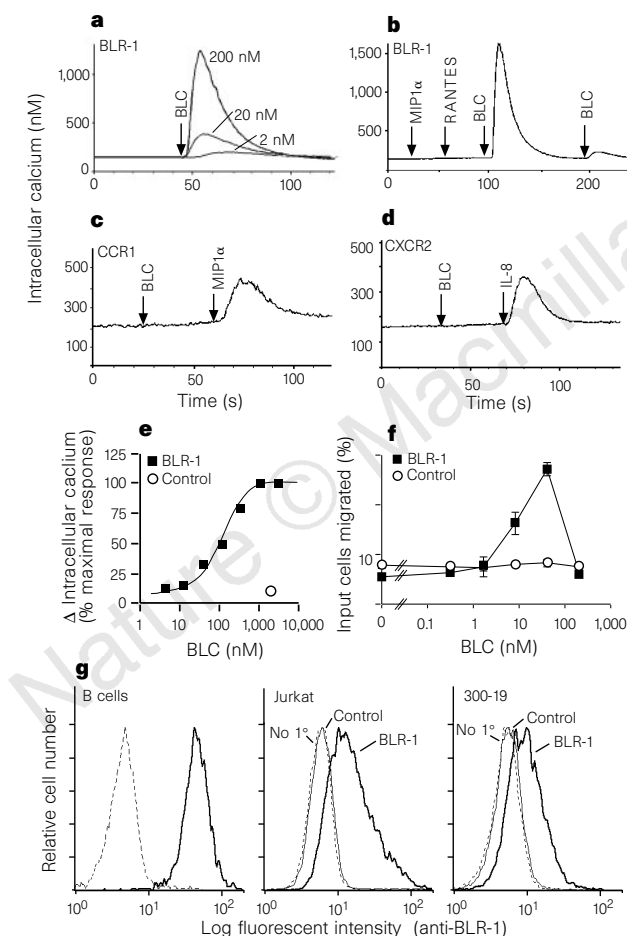


Figure 4 BLR-1 mediated calcium mobilization and chemotaxis in response to BLC. HEK 293 cells, transfected with the indicated chemokine receptors (**a–d**), were loaded with Indo-1 and assayed by spectrofluorimetry for changes in intracellular calcium levels over time in response to BLC. **a**, Calcium flux as a function of BLC concentration (nM). **b**, Specificity of the response of BLR-1 to BLC. **c**, Lack of response of CCR1-transfected cells to BLC. **d**, Lack of response of CXCR2-transfected cells to BLC. **e**, Percentage of maximal calcium flux as a function of BLC concentration in BLR-1-transfected 300-19 cells. **f**, Chemotactic response of BLR-1-transfected Jurkat cells to BLC. Results are expressed as in Fig. 3. **g**, Flow-cytometric analysis of BLR-1 expression on spleen B cells and BLR-1-transfected (BLR-1) Jurkat and 300-19 cells. Non-transfected (control) cells and background fluorescence in the absence of primary antibody (No. 1°, dotted lines) are shown.

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Correspondence and requests for materials should be addressed to J.G.C. (e-mail: cyster@itsa.ucsf.edu). The mouse and human BLC sequences are deposited in the Genbank database under accession numbers AF044196 and AF044197.

Direct activation of inward rectifier potassium channels by PIP₂ and its stabilization by Gβγ

Chou-Long Huang*, Siyi Feng† & Donald W. Hilgemann†

* Department of Medicine, Division of Nephrology, and † Department of Physiology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235, USA

Inward rectifier K⁺ channels, which modulate electrical activity in many cell types, are regulated by protein kinases^{1,2}, guanine-nucleotide-binding proteins (G proteins)^{3–6} and probably actin cytoskeleton⁷. Generation of phosphatidylinositol 4,5-bisphosphate (PIP₂) by ATP-dependent lipid kinases is known to activate inward rectifier K⁺ channels in cardiac membrane patches⁸. Here we report that several cloned inward rectifier K⁺ channels directly bind PIP₂, and that this binding correlates with channel

activity. Application of ATP or PIP₂ liposomes activates the cloned channels. Stabilized by lipid phosphatase inhibitors, PIP₂ antibodies⁹ potentially inhibit each channel with a unique rate (GIRK1/4 (refs 3–5) ≈ GIRK2 (ref. 6) ≫ IRK1 (ref. 10) ≈ ROMK (ref. 11)). Consistent with the faster dissociation of PIP₂ from the GIRK channels, the carboxy terminus of GIRK1 binds ³H-PIP₂ liposomes more weakly than does that of IRK1 or ROMK1. Mutation of a conserved arginine to glutamine at position 188 reduces the ability of ROMK1 to bind PIP₂ and increases its sensitivity to inhibition by PIP₂ antibodies. Interactions between GIRK channels and PIP₂ are modulated by the βγ subunits of the G protein (Gβγ). When GIRK1/4 channels are allowed to run down completely, they are not activated by addition of Gβγ alone, but application of PIP₂ activates them in minutes without Gβγ and in just seconds with Gβγ. Finally, coexpression of Gβγ with GIRK channels slows the inhibition of K⁺ currents by PIP₂ antibodies by more than 10-fold. Thus Gβγ activates GIRK channels by stabilizing interactions between PIP₂ and the K⁺ channel.

We used the *Xenopus* oocyte expression system to examine the possible role of PIP₂ in the cloned inward rectifier channels IRK1, ROMK1, GIRK1/4 and GIRK2. In giant excised membrane patches⁸, we studied current–voltage (*I*–*V*) relations (Fig. 1a) and inward K⁺ currents at –30 mV (Fig. 1b, e) with 100 mM K⁺ on both sides of the membrane. Na⁺ (30 mM) was included on the cytoplasmic side because GIRK1/4 channels are partly activated by Na⁺, and GIRK2 channels require Na⁺ (refs 6, 12). As reported previously^{6,13}, the G-protein-coupled inward rectifier K⁺ channels, GIRK1/4 and GIRK2, express significant basal K⁺ currents without receptor stimulation or exogenous Gβγ (Fig. 1a, d).

Rundown of the inward rectifier currents in giant patches was prevented for the most part by including fluoride (5 mM F[–]),

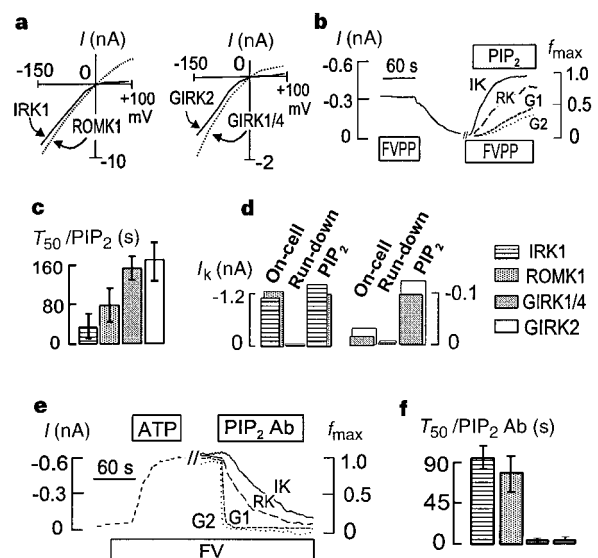


Figure 1 PIP₂ dependence of cloned inward rectifier K⁺ channels. **a**, *I*–*V* relations of IRK1 (IK), ROMK1 (RK), GIRK1/4 (G1) and GIRK2 (G2) K⁺ channels in the 'on-cell' configuration. **b**, Rundown of inward rectifier channels and recovery by application of PIP₂. The rundown record (solid line) is from an IRK1 patch. **c**, Time to half-maximal activation (*T*₅₀) during application of PIP₂ (mean ± s.e.m., *n* = 3–5). **d**, Inward K⁺ currents in the on-cell configuration, after rundown and after application of PIP₂. **e**, Activation of inward rectifier K⁺ channels by cytoplasmic ATP and inhibition by PIP₂ antibodies. The record is for a patch expressing GIRK1/4 channels, but similar results were obtained with the other channels. Channel activities were stable in FV solution after removal of ATP, and were completely inhibited by subsequent application of PIP₂ antibody. Results are normalized. **f**, Time to half-maximal inhibition by PIP₂ antibody (mean ± s.e.m., *n* = 3–5).

vanadate (0.1 mM VO_4) and pyrophosphate (10 mM PP_i) in cytoplasmic solutions (FVPP solution), probably because it inhibited lipid phosphatases¹⁴. Changing the cytoplasmic solution to one including 0.5 mM free Mg^{2+} induced channel rundown (Fig. 1b). Then application of PIP_2 (50 μM) activated channels with a channel-specific time course (Fig. 1b, c). In the presence of FVPP, the average half-times (T_{50}) for activation were 38 and 77 s for IRK1 and ROMK1 channels, and >2 min for GIRK1/4 and GIRK2 channels (Fig. 1c). Without FVPP, PIP_2 was often ineffective, suggesting that membrane-associated lipid phosphatases are very active in oocyte patches.

For IRK1 and ROMK1 channels, the conductances measured 'on-cell' (before excision) were similar to those achieved with PIP_2 after channel rundown (Fig. 1d). For both GIRK channels, expressed without $\text{G}\beta\gamma$ subunits, on-cell conductances were much smaller than those achieved with PIP_2 (Fig. 1d), and the longer activation times suggest that more PIP_2 is required for GIRK channel activation. Without $\text{G}\beta\gamma$, therefore, GIRK channels are only weakly activated by prevailing PIP_2 concentrations in cells.

In the presence of fluoride (0.2 mM) and vanadate (0.1 mM) (FV solution), Mg-ATP (2 mM) activated GIRK1/4 (Fig. 1e), as well as

the other channels (not shown). As expected if ATP is acting to generate PIP_2 , PIP_2 -specific monoclonal antibodies completely inhibited each channel type (Fig. 1e, f). Control mouse IgG and other antibodies had no effect (not shown), previous application of PIP_2 prevented inhibition by antibody, and other tests for the involvement of PIP_2 in the effect of ATP⁸ were positive. The rapid ATP responses suggest that lipid kinases, as well as phosphatases, are active in oocyte patches. Because application of PIP_2 after the antibody did not reactivate channels (not shown), stable complexes may be formed between PIP_2 , inward rectifier channels, and PIP_2 antibodies, as described previously for other PIP_2 -binding proteins¹⁵. Average T_{50} values for inhibition of each channel type by PIP_2 antibodies are shown in Fig. 1f. A >10 -fold faster inhibition of GIRK1/4 and GIRK2, compared with ROMK1 and IRK1, suggests that PIP_2 is much more easily bound by antibody at GIRK channels and that GIRK channels have a lower affinity for PIP_2 than IRK1 and ROMK1 channels.

Consistent with a direct mechanism of PIP_2 action, we found that the C terminus of each channel type bound PIP_2 , and that the fusion proteins of glutathione *S*-transferase and the C-terminus of ROMK1 (GST-RKC) and IRK1 (GST-IKC) bound $^3\text{H-PIP}_2$ liposomes stronger than that with the C terminus of GIRK1 (GST-GKC) (Fig. 2a). The control GST fusion protein, the N terminus of IRK1 (GST-IKN) (Fig. 2a) and the N terminus of GIRK1 (GST-GKN; not shown) did not bind PIP_2 . The binding of $^3\text{H-PIP}_2$ to GST-RKC could be competed away by excess unlabelled PIP_2 , but not by phosphatidylcholine, giving an apparent K^d of

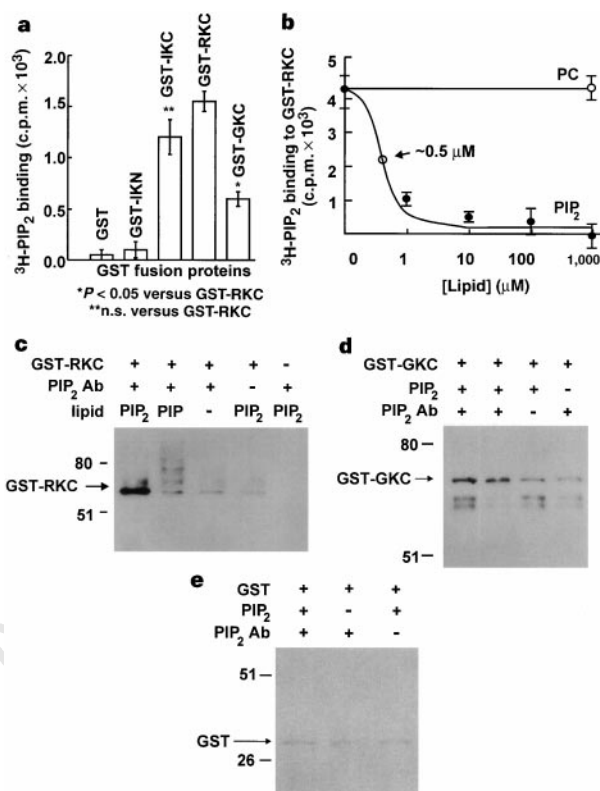


Figure 2 Direct binding of PIP_2 with inward rectifier K^+ channels. **a**, Binding (mean $\pm$ s.e.m., $n = 3$) of $^3\text{H-PIP}_2$ liposomes (0.3 μM) by GST-IKC, GST-RKC and GST-GKC, but not by GST-IKN or GST alone (100 nM each). Background binding of $^3\text{H-PIP}_2$ to the glutathione 4B sepharose beads (ranges ~15–25% of the total binding to GST-RKC) was subtracted. * $P < 0.05$ versus GST-RKC; **n.s. **b**, Displacement of the binding of $^3\text{H-PIP}_2$ (1 μM) to GST-RKC (100 nM) by unlabelled PIP_2 , but not by phosphatidylcholine (PC). Each circle represents mean $\pm$ s.e.m. ($n = 3$) of the $^3\text{H-PIP}_2$ bound to GST-RKC in the presence of 0, 1, 10, 100 or 1,000 μM of unlabelled PIP_2 (filled circles) or 1,000 μM of PC (open circle). **c**, GST-RKC (100 nM) was incubated with lipid vesicles (25% PIP_2 or PIP with 75% PC) for co-precipitation by PIP_2 antibodies. Arrow indicates GST-RKC detected by polyclonal antibody against amino acids 360–391 of ROMK1. **d**, GST-GKC (100 nM) was incubated with 25% PIP_2 vesicles for co-precipitation by PIP_2 antibodies. Lanes 1 and 2 (from left) are duplicates. Arrow indicates GST-GKC detected by polyclonal antibody against amino acids 364–375 of GIRK1. **e**, Arrow indicates GST detected by monoclonal antibody against GST (Santa Cruz).

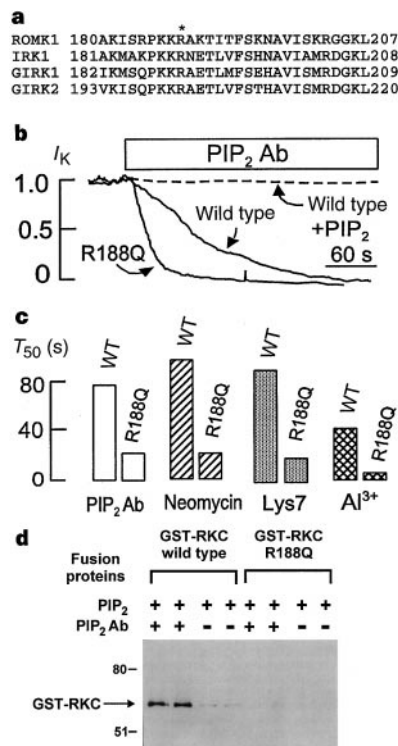


Figure 3 **a**, Amino-acid alignment for the proximal C-terminal regions of the inward rectifier K^+ channels. The numbers indicate positions of amino acids for each channel. Arg 188 of ROMK1 (indicated by asterisk) is conserved among these channels. **b**, Inhibition of wild-type ROMK channels currents and R188Q mutant currents by PIP_2 antibody in FV solution. **c**, T_{50} for inhibition of wild-type ROMK1 (WT) and R188Q mutant channels currents by PIP_2 antibody ($n = 4$), neomycin ($n = 2$), heptalysine (Lys 7; $n = 2$) or Al^{3+} (50 μM) plus EGTA (10 mM) ($n = 2$). **d**, Co-precipitation of PIP_2 vesicles (in duplicate) with the wild-type GST-RKC (lanes 1 and 2) but not with the R188Q mutant GST-RKC (lanes 5 and 6). Lanes 3, 4, 7 and 8 serve as controls (background bindings).

~0.5 μM (Fig. 2b). Because PIP₂ antibodies can immunoprecipitate some PIP₂-protein complexes¹⁵, we studied the direct interaction between PIP₂ and ROMK1 by co-immunoprecipitation of GST-RKC with PIP₂-containing vesicle using PIP₂ antibodies (Fig. 2c). We found that GST-RKC co-precipitated with PIP₂ vesicles by PIP₂ antibodies ~10 times more than the background level (Fig. 2c, lane 1 from left versus lanes 2–4). The absolute requirement of PIP₂ and PIP₂ antibodies for precipitation of GST-RKC indicates a specific interaction between PIP₂ and GST-RKC. To a lesser degree, GST-GKC co-precipitated with PIP₂ by PIP₂ antibodies (~2–3 times over background; Fig. 2d, lanes 1 and 2 versus lanes 3 and 4). The control GST protein did not co-precipitate with PIP₂ by the antibodies (Fig. 2e).

The binding and activation of inward rectifier K⁺ channels by the anionic PIP₂ suggests that the region of conserved cationic residues in the proximal C-terminal hydrophilic domain of the channels can be considered as a putative PIP₂-binding site¹⁶ (Fig. 3a). Deletion of this region (amino acids 180–207) from GST-RKC markedly reduced its ability to bind PIP₂ (not shown). Mutation of one conserved arginine to glutamine at position 188 (R188Q) in ROMK1 decreased the apparent 'on-cell' K⁺ conductance while not affecting current-voltage relations (not shown). In excised patches, the mutant channels were inhibited much more rapidly by PIP₂ antibodies (Fig. 3b, c), indicating that the mutant has a

lower affinity for PIP₂. The T_{50} for inhibition by antibody was 75 ± 18 s (s.e.m.) for wild-type ROMK1 and 13 ± 4 s for the R188Q mutant ($P < 0.001$). Excess PIP₂ abolished subsequent effects of the antibody, consistent with a specific PIP₂ antibody effect. Other agents that bind PIP₂ (neomycin¹⁷, heptalysine^{8,18} and aluminium⁸) also inhibited the mutant channel activity faster than wild-type channels (Fig. 3c). Consistent with a role for Arg 188 in binding PIP₂, an R188Q mutant of GST-RKC did not co-precipitate significantly with PIP₂ vesicles (Fig. 3d). These results validate the use of PIP₂ antibody to assess the relative affinities of channels for PIP₂, and support the idea that PIP₂ is bound cooperatively by multiple cationic residues of the channels, similar to the binding of PIP₂ to the pleckstrin homology (PH) domains¹⁹.

G $\beta\gamma$ activates GIRK1/4 (refs 5, 13, 20) and GIRK2 (ref. 6) channels by direct binding to the channels^{21–23}, but it is not known how binding of G $\beta\gamma$ leads to channel activation. Application of PIP₂ to GIRK1/4 without exogenous G $\beta\gamma$ slowly increased channel activity over a few minutes to a high, supra-basal level (Fig. 1a, b). This level could not be increased by addition of G $\beta\gamma$ (3 nM) or decreased by application of 15 nM G α_{i1} (Fig. 4a, b). Thus PIP₂ can maximally activate GIRK channels without G $\beta\gamma$.

To examine how the activation mechanisms of PIP₂ and G $\beta\gamma$ are related, membrane patches were allowed to run down for 10 min in Mg²⁺-containing solutions to deplete them completely of PIP₂. Addition of G $\beta\gamma$ to these patches had almost no effect (Fig. 4c). Addition of PIP₂ (50 μM) after G $\beta\gamma$ strongly activated channels in just a few seconds, compared with 60–300 s without G $\beta\gamma$ (Fig. 4a, c), indicating that low concentrations of PIP₂ are essential for the activation of GIRK1/4 channels by G $\beta\gamma$. Sequestering of G $\beta\gamma$ by G α_{i1} inhibited the current in <5 s, indicating that the fast activation of channels by PIP₂ required preapplication of G $\beta\gamma$. As with the experiments shown in Fig. 4a, b, the channels could be fully activated in minutes by a second application of PIP₂ during which a role of G $\beta\gamma$ was blocked by G α_{i1} . As with K_{ATP} channels and Na⁺, Ca²⁺ exchangers⁸, aluminum-EGTA (50 μM) reversed the stimulation, probably by sequestering PIP₂. As in previous reports^{5,13,20}, G $\beta\gamma$ was capable of fully activating GIRK1/4 channels if they were not allowed to run down completely (Fig. 4d), such that PIP₂ had no further effect. Thus residual PIP₂ is sufficient to support G $\beta\gamma$ action, and we predict that changes in PIP₂ concentration would modulate the effectiveness of G $\beta\gamma$. Results with PIP₂ antibodies verify that PIP₂ is essential for G $\beta\gamma$ activation of channels and that G $\beta\gamma$ stabilizes PIP₂-channel interactions. Application of recombinant G $\beta\gamma$ markedly slowed the inhibition of channels by PIP₂ antibodies (Fig. 4e). When GIRK1/4 channels were activated by overexpression of G $\beta\gamma$ in oocytes, inhibition by PIP₂ antibodies was still several-fold slower (Fig. 4e). Similar results were obtained with GIRK2 channels (Fig. 4f).

Ion channels can now be added to the growing list of proteins that bind PIP₂ (refs 24–26). The synergistic activation of GIRK channels by PIP₂ and G $\beta\gamma$ is reminiscent of β -adrenergic receptor kinase (β -ARK1) activation by PIP₂ and G $\beta\gamma$ ²⁶. If a region of the C-terminal hydrophilic domain of GIRK channels forms part of the pore²⁷, the increase in binding of the C terminus to membrane PIP₂ by G $\beta\gamma$ may stabilize the open pore. Consistent with this tethering mechanism, we found that G $\beta\gamma$ increases the association between GST-GKC and PIP₂-containing vesicles (not shown). It is not yet known whether other G $\beta\gamma$ effectors²⁸ also interact with PIP₂ and whether G $\beta\gamma$ actions are modulated by phosphatidylinositol signalling mechanisms in cells. □

Methods

Electrophysiology. The giant patch methods (see ref. 8) and *in vitro* transcription of mCAP cRNA²¹ have been described. Extracellular (pipette) solution contained (in mM) 100 KCl, 2 CaCl₂, 20 HEPES and 0.1 ouabain. Cytoplasmic (bath) solution contained 100 KCl, 20 HEPES, 10 EGTA and 0.5 MgCl₂. FVPP solution, which chelates Mg²⁺, also contained 5 NaF, 0.1 Na₃VO₄

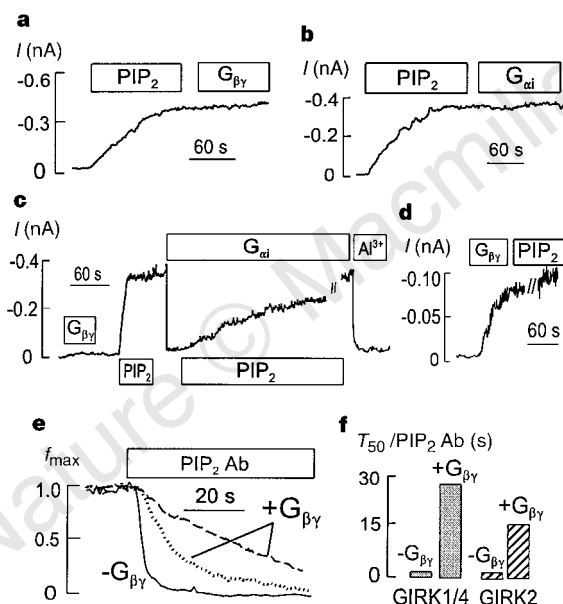


Figure 4 G $\beta\gamma$ -induced sensitization of GIRK1/4 channels to activation by PIP₂. **a**, Activation of GIRK1/4 channels by PIP₂ precludes further activation by G $\beta\gamma$. GIRK1/4 channels expressed without G $\beta\gamma$ were allowed to run down to just detectable levels, and then PIP₂ was applied in the presence of FVPP. After maximal activation was achieved (3 min), G $\beta\gamma$ had no effect. **b**, GIRK1/4 channels fully activated by PIP₂ (as in **a**) are insensitive to G α_{i1} . **c**, G $\beta\gamma$ -induced sensitization of GIRK1/4 channels to activation by PIP₂. GIRK1/4 current was allowed to run down for 10 min in the presence of 4 mM Mg²⁺ to deplete PIP₂. The experiment was then performed with FVPP. Record shown is representative of 3 similar experiments. **d**, Activation of GIRK1/4 channels by G $\beta\gamma$ precludes further activation by PIP₂. Current was allowed to run down to just detectable levels, and then G $\beta\gamma$ was applied in the presence of FVPP. After maximal activation was achieved, PIP₂ had no effect. **e**, Inhibition of GIRK1/4 currents by PIP₂ antibody. Currents were normalized to their magnitudes before PIP₂ antibody was applied. Without G $\beta\gamma$ (solid line), current was inhibited by 50% in 2 s or less. With G $\beta\gamma$ preapplied (dotted line), 50% inhibition was 12 s. With coexpression of G $\beta\gamma$ subunits (dashed line), 50% inhibition occurred at 35 s. **f**, T_{50} for inhibition by PIP₂ antibody for GIRK1/4 or GIRK2 channels without and with coexpression of G $\beta\gamma$ ($n = 3$ for each column).

and 10 Na₃HP₂O₇. FV solution also contained 0.2 NaF and 0.1 Na₃VO₄. Rarely, irreversible current rundown still occurred with FVPP. The total Na⁺ concentration of all cytoplasmic solutions was adjusted to 30 mM with NaOH, and pH was adjusted to 7.0 with *N*-methylglucamine (NMG) or HCl. PIP₂ liposomes (20–200 nm) were prepared by sonicating 1 mM PIP₂ (Boehringer Mannheim) in distilled water. Reconstituted monoclonal PIP₂ antibody (Perspective Biosystems, Framingham, MA) was diluted 40-fold into experimental solution. Current–voltage relations of all currents reversed at *E*_K and showed characteristic rectification, mostly owing to the presence of Na⁺ in FVPP and possibly also residual polyamines. Current records presented (measured at 30 °C, –30 mV holding potential) are digitized strip-chart recordings. Purified bovine brain Gβγ²⁹ was diluted just before application such that the final detergent (CHAPS) concentration was 5 μM. Detergent-containing solution was washed away thoroughly before application of PIP₂, because application of phospholipid vesicles in the presence of detergent usually reversed the effects of Gβγ; presumably, Gβγ can be extracted from membranes by detergent plus phospholipids.

Molecular biology. R188Q mutation was constructed by insertion of the mutant oligonucleotides between the *Bsm*I and *Bgl*II sites of pSPORT–ROMK1 (ref. 11). A polymerase chain reaction (PCR) fragment (amino acids 180–391) from pSPORT–ROMK1 R188Q mutant was subcloned into pGEX-2T vector (Pharmacia) for expression of R188Q mutant protein of GST–RKC. The construction, expression and purification of GST–IKC (amino acids 182–428 of IRK1), GST–GKC (180–462 of GIRK1), GST–IKN (1–86 of IRK1) have been described^{21,22}.

In vitro PIP₂ binding assay. ³H-PIP₂ in chloroform-methanol (1:1) (American Radiolabeled Chemicals; 0.4 μCi nM^{–1} specific activity) was dried under N₂ and sonicated in 100 μl phosphate buffered saline (PBS) to form pure ³H-PIP₂ liposomes. Purified GST fusion protein (100 nM) was incubated with ³H-PIP₂ (0.2–1 μM) and precipitated by glutathione 4B-Sepharose beads. After 1 wash with PBS, the precipitates were dissolved in SDS gel loading buffer and counted in a beta-scintillation counter using a window for ³H. The bound ³H radioactivity was typically in the range ~2–8% of the total added. For co-immunoprecipitation, 25% PIP₂ or PIP in 75% phosphatidylcholine (PC) background (30 μg PIP₂ or PIP (Boehringer Mannheim) and 90 μg phosphatidylcholine (Sigma)), both in chloroform, were dried down together and sonicated in 300 μl PBS to form mixed liposome. GST fusion proteins were first incubated with 25% PIP₂ or PIP liposome (100 μM) and PIP₂ antibodies (1:100 dilution) for 2 h and with protein A-Sepharose for a further 30 min. After one wash with PBS, the immunoprecipitates were separated by 10% SDS–PAGE, probed with specific antibodies^{21,22}, and visualized by ECL (Amersham). Each experiment was performed at least twice with similar results. The relative amount of immunoreactivity in each lane was quantified by serial dilutions of sample²¹.

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Correspondence and requests for materials should be addressed to C.L.H. (e-mail: chuan1@mednet.swmed.edu).

Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*

Andrew Fire*, SiQun Xu*, Mary K. Montgomery*, Steven A. Kostas*†, Samuel E. Driver‡ & Craig C. Mello‡

* Carnegie Institution of Washington, Department of Embryology, 115 West University Parkway, Baltimore, Maryland 21210, USA

† Biology Graduate Program, Johns Hopkins University,

3400 North Charles Street, Baltimore, Maryland 21218, USA

‡ Program in Molecular Medicine, Department of Cell Biology, University of Massachusetts Cancer Center, Two Biotech Suite 213, 373 Plantation Street, Worcester, Massachusetts 01605, USA

Experimental introduction of RNA into cells can be used in certain biological systems to interfere with the function of an endogenous gene^{1,2}. Such effects have been proposed to result from a simple antisense mechanism that depends on hybridization between the injected RNA and endogenous messenger RNA transcripts. RNA interference has been used in the nematode *Caenorhabditis elegans* to manipulate gene expression^{3,4}. Here we investigate the requirements for structure and delivery of the interfering RNA. To our surprise, we found that double-stranded RNA was substantially more effective at producing interference than was either strand individually. After injection into adult animals, purified single strands had at most a modest effect, whereas double-stranded mixtures caused potent and specific interference. The effects of this interference were evident in both the injected animals and their progeny. Only a few molecules of injected double-stranded RNA were required per affected cell, arguing against stoichiometric interference with endogenous

mRNA and suggesting that there could be a catalytic or amplification component in the interference process.

Despite the usefulness of RNA interference in *C. elegans*, two features of the process have been difficult to explain. First, sense and antisense RNA preparations are each sufficient to cause interference^{3,4}. Second, interference effects can persist well into the next generation, even though many endogenous RNA transcripts are rapidly degraded in the early embryo⁵. These results indicate a fundamental difference in behaviour between native RNAs (for example, mRNAs) and the molecules responsible for interference. We sought to test the possibility that this contrast reflects an underlying difference in RNA structure. RNA populations to be injected are

generally prepared using bacteriophage RNA polymerases⁶. These polymerases, although highly specific, produce some random or ectopic transcripts. DNA transgene arrays also produce a fraction of aberrant RNA products³. From these facts, we surmised that the interfering RNA populations might include some molecules with double-stranded character. To test whether double-stranded character might contribute to interference, we further purified single-stranded RNAs and compared interference activities of individual strands with the activity of a deliberately prepared double-stranded hybrid.

The *unc-22* gene was chosen for initial comparisons of activity. *unc22* encodes an abundant but nonessential myofilament protein⁷⁻⁹. Several thousand copies of *unc-22* mRNA are present in each

Table 1 Effects of sense, antisense and mixed RNAs on progeny of injected animals

Gene	segment	Size (kilobases)	Injected RNA	F ₁ phenotype
<i>unc-22</i>				<i>unc-22</i> -null mutants: strong twitchers ^{7,8}
<i>unc22A</i> *	Exon 21-22	742	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
<i>unc22B</i>	Exon 27	1,033	Sense	Wild type
			Antisense	Wild type
			Sense + antisense	Strong twitchers (100%)
<i>unc22C</i>	Exon 21-22†	785	Sense + antisense	Strong twitchers (100%)
<i>fem-1</i>				<i>fem-1</i> -null mutants: femal (no sperm) ¹³
<i>fem1A</i>	Exon 10‡	531	Sense	Hermaphrodite (98%)
			Antisense	Hermaphrodite (>98%)
			Sense + antisense	Female (72%)
<i>fem1B</i>	Intron 8	556	Sense + antisense	Hermaphrodite (>98%)
<i>unc-54</i>				<i>unc-54</i> -null mutants: paralysed ²¹
<i>unc54A</i>	Exon 6	576	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
<i>unc54B</i>	Exon 6	651	Sense	Wild type (100%)
			Antisense	Wild type (100%)
			Sense + antisense	Paralysed (100%)§
<i>unc54C</i>	Exon 1-5	1,015	Sense + antisense	Arrested embryos and larvae (100%)
<i>unc54D</i>	Promoter	567	Sense + antisense	Wild type (100%)
<i>unc54E</i>	Intron 1	369	Sense + antisense	Wild type (100%)
<i>unc54F</i>	Intron 3	386	Sense + antisense	Wild type (100%)
<i>hlh-1</i>				<i>hlh-1</i> -null mutants: lumpy-dumpy larvae ¹⁶
<i>hlh1A</i>	Exons 1-6	1,033	Sense	Wild type (<2% lpy-dpy)
			Antisense	Wild type (<2% lpy-dpy)
			Sense + antisense	Lpy-dpy larvae (>90%)
<i>hlh1B</i>	Exons 1-2	438	Sense + antisense	Lpy-dpy larvae (>80%)
<i>hlh1C</i>	Exons 4-6	299	Sense + antisense	Lpy-dpy larvae (>80%)
<i>hlh1D</i>	Intron 1	697	Sense + antisense	Wild type (<2% lpy-dpy)
<i>myo-3</i> -driven GFP transgenes†				Makes nuclear GFP in body muscle
<i>myo-3::NLS::gfp::lacZ</i>				Nuclear GFP-LacZ pattern of parent strain
<i>gfpG</i>	Exons 2-5	730	Sense	Nuclear GFP-LacZ pattern of parent strain
			Antisense	Nuclear GFP-LacZ absent in 98% of cells
			Sense + antisense	Nuclear GFP-LacZ absent in >95% of cells
<i>lacZL</i>	Exon 12-14	830	Sense + antisense	Nuclear GFP-LacZ absent in >95% of cells
<i>myo-3::MtLS::gfp</i>				Makes mitochondrial GFP in body muscle
<i>gfpG</i>	Exons 2-5	730	Sense	Mitochondrial-GFP pattern of parent strain
			Antisense	Mitochondrial-GFP pattern of parent strain
			Sense + antisense	Mitochondrial-GFP absent in 98% of cells
<i>lacZL</i>	Exon 12-14	830	Sense + antisense	Mitochondrial-GFP pattern of parent strain

Each RNA was injected into 6-10 adult hermaphrodites ($0.5 \times 10^6 - 1 \times 10^6$ molecules into each gonad arm). After 4-6 h (to clear prefertilized eggs from the uterus), injected animals were transferred and eggs collected for 20-22 h. Progeny phenotypes were scored upon hatching and subsequently at 12-24 h intervals.

* to obtain a semiquantitative assessment of the relationship between RNA dose and phenotypic response, we injected each *unc22A* RNA preparation at a series of different concentrations (see figure in Supplementary information for details). At the highest dose tested (3.6×10^6 molecules per gonad), the individual sense and antisense *unc22A* preparations produced some visible twitching (1% and 11% of progeny, respectively). Comparable doses of double-stranded *unc22A* RNA produced visible twitching in all progeny, whereas a 120-fold lower dose of double-stranded *unc22A* RNA produced visible twitching in 30% of progeny. † *unc22C* also carries the 43-nucleotide intron between exons 21 and 22. ‡ *fem1A* carries a portion (131 nucleotides) of intron 10. § Animals in the first affected broods (laid 4-24 h after injection) showed movement defects indistinguishable from those of *unc-54*-null mutants. A variable fraction of these animals (25%-75%) failed to lay eggs (another phenotype of *unc-54*-null mutants), whereas the remainder of the paralysed animals did lay eggs. This may indicate incomplete interference with *unc-54* activity in vulval muscles. Animals from later broods frequently show a distinct partial loss-of-function phenotype, with contractility in a subset of body-wall muscles. || Phenotypes produced by RNA-mediated interference with *hlh-1* included arrested embryos and partially elongated L1 larvae (the *hlh-1*-null phenotype). These phenotypes were seen in virtually all progeny after injection of double-stranded *hlh1A* and in about half of the affected animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*. A set of less severe defects was seen in the remainder of the animals produced after injection of double-stranded *hlh1B* and double-stranded *hlh1C*. The less severe phenotypes are characteristic of partial loss of function of *hlh-1* (B. Harfe and A.F., unpublished observations). ‡ the host for these injections, strain PD4251, expresses both mitochondrial GFP and nuclear GFP-LacZ (see Methods). This allows simultaneous assay for interference with *gfp* (seen as loss of all fluorescence) and with *lacZ* (loss of nuclear fluorescence). The table describes scoring of animals as L1 larvae. Double-stranded *gfpG* caused a loss of GFP in all but 0-3 of the 85 body muscles in these larvae. As these animals mature to adults, GFP activity was seen in 0-5 additional body-wall muscles and in the 8 vulval muscles. Lpy-dpy, lumpy-dumpy.

striated muscle cell³. Semiquantitative correlations between *unc-22* activity and phenotype of the organism have been described⁸: decreases in *unc-22* activity produce an increasingly severe twitching phenotype, whereas complete loss of function results in the additional appearance of muscle structural defects and impaired motility.

Purified antisense and sense RNAs covering a 742-nucleotide segment of *unc-22* had only marginal interference activity, requiring a very high dose of injected RNA to produce any observable effect (Table 1). In contrast, a sense–antisense mixture produced highly effective interference with endogenous gene activity. The mixture was at least two orders of magnitude more effective than either single strand alone in producing genetic interference. The lowest dose of the sense–antisense mixture that was tested, ~60,000 molecules of each strand per adult, led to twitching phenotypes in an average of 100 progeny. Expression of *unc-22* begins in embryos

containing ~500 cells. At this point, the original injected material would be diluted to at most a few molecules per cell.

The potent interfering activity of the sense–antisense mixture could reflect the formation of double-stranded RNA (dsRNA) or, conceivably, some other synergy between the strands. Electrophoretic analysis indicated that the injected material was predominantly double-stranded. The dsRNA was gel-purified from the annealed mixture and found to retain potent interfering activity. Although annealing before injection was compatible with interference, it was not necessary. Mixing of sense and antisense RNAs in low-salt concentrations (under conditions of minimal dsRNA formation) or rapid sequential injection of sense and antisense strands were sufficient to allow complete interference. A long interval (>1 h) between sequential injections of sense and antisense RNA resulted in a dramatic decrease in interfering activity. This suggests that injected single strands may be degraded or otherwise rendered inaccessible in the absence of the opposite strand.

A question of specificity arises when considering known cellular responses to dsRNA. Some organisms have a dsRNA-dependent protein kinase that activates a panic-response mechanism¹⁰. Conceivably, our sense–antisense synergy might have reflected a non-specific potentiation of antisense effects by such a panic mechanism. This is not the case: co-injection of dsRNA segments unrelated to *unc-22* did not potentiate the ability of single *unc-22*-RNA strands to mediate inhibition (data not shown). We also investigated whether double-stranded structure could potentiate interference activity when placed in *cis* to a single-stranded segment. No such potentiation was seen: unrelated double-stranded sequences located 5' or 3' of a single-stranded *unc-22* segment did not stimulate interference. Thus, we have only observed potentiation of interference when dsRNA sequences exist within the region of homology with the target gene.

The phenotype produced by interference using *unc-22* dsRNA was extremely specific. Progeny of injected animals exhibited behaviour that precisely mimics loss-of-function mutations in *unc-22*. We assessed target specificity of dsRNA effects using three additional genes with well characterized phenotypes (Fig. 1, Table 1). *unc-54* encodes a body-wall-muscle heavy-chain isoform of myosin that is required for full muscle contraction^{7,11,12}; *fem-1* encodes an ankyrin-repeat-containing protein that is required in hermaphrodites for sperm production^{13,14}; and *hlh-1* encodes a *C. elegans* homologue of myoD-family proteins that is required for proper body shape and motility^{15,16}. For each of these genes, injection of related dsRNA produced progeny broods exhibiting

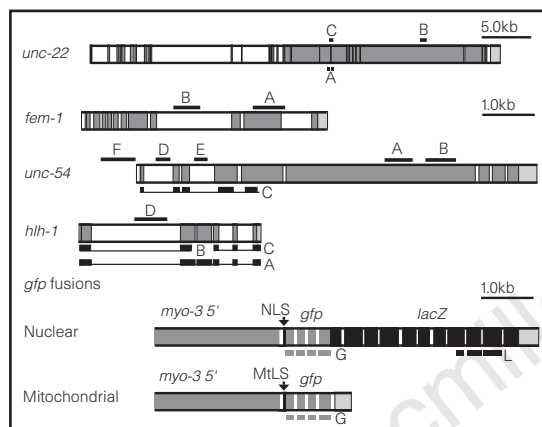


Figure 1 Genes used to study RNA-mediated genetic interference in *C. elegans*. Intron–exon structure for genes used to test RNA-mediated inhibition are shown (grey and filled boxes, exons; open boxes, introns; patterned and striped boxes, 5' and 3' untranslated regions). *unc-22*, ref. 9, *unc-54*, ref. 12, *fem-1*, ref. 14, and *hlh-1*, ref. 15). Each segment of a gene tested for RNA interference is designated with the name of the gene followed by a single letter (for example, *unc-22C*). These segments are indicated by bars and upper-case letters above and below each gene. Segments derived from genomic DNA are shown above the gene; segments derived from cDNA are shown below the gene. NLS, nuclear-localization sequence; MtLS, mitochondrial localization sequence.

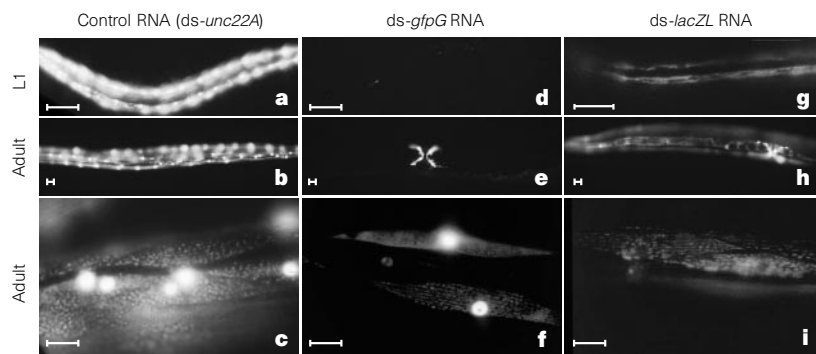


Figure 2 Analysis of RNA-interference effects in individual cells. Fluorescence micrographs show progeny of injected animals from GFP-reporter strain PD4251. **a–c**, Progeny of animals injected with a control RNA (double-stranded (ds)-*unc22A*). **a**, Young larva, **b**, adult, **c**, adult body wall at high magnification. These GFP patterns appear identical to patterns in the parent strain, with prominent fluorescence in nuclei (nuclear-localized GFP–LacZ) and mitochondria (mitochondrially targeted GFP). **d–f**, Progeny of animals injected with ds-*gfpG*. Only a single active cell is seen in the larva in **d**, whereas the entire vulval

musculature expresses active GFP in the adult animal in **e, f**. Two rare GFP-positive cells in an adult: both cells express both nuclear-targeted GFP–LacZ and mitochondrial GFP. **g–i**, Progeny of animals injected with ds-*lacZL* RNA: mitochondrial-targeted GFP seems unaffected, while the nuclear-targeted GFP–LacZ is absent from almost all cells (for example, see larva in **g**). **h**, A typical adult, with nuclear GFP–LacZ lacking in almost all body-wall muscles but retained in vulval muscles. Scale bars represent 20 μm.

the known null-mutant phenotype, whereas the purified single RNA strands produced no significant interference. With one exception, all of the phenotypic consequences of dsRNA injection were those expected from interference with the corresponding gene. The exception (segment *unc54C* which led to an embryonic- and larval-arrest phenotype not seen with *unc-54*-null mutants) was illustrative. This segment covers the highly conserved myosin-motor domain, and might have been expected to interfere with activity of other highly related myosin heavy-chain genes¹⁷. The *unc54C* segment has been unique in our overall experience to date: effects of 18 other dsRNA segments (Table 1; and our unpublished observations) have all been limited to those expected from previously characterized null mutants.

The pronounced phenotypes seen following dsRNA injection indicate that interference effects are occurring in a high fraction of

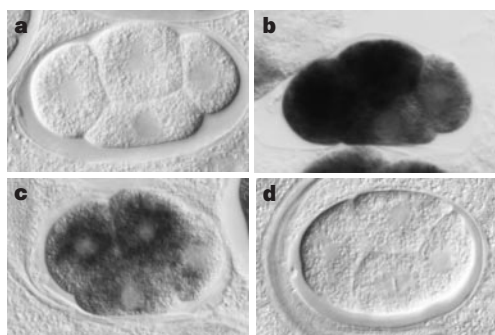


Figure 3 Effects of *mex-3* RNA interference on levels of the endogenous mRNA. Interference contrast micrographs show *in situ* hybridization in embryos. The 1,262-nt *mex-3* cDNA clone²⁰ was divided into two segments, *mex-3A* and *mex-3B*, with a short (325-nt) overlap (similar results were obtained in experiments with no overlap between interfering and probe segments). *mex-3B* antisense or dsRNA was injected into the gonads of adult animals, which were fed for 24 h before fixation and *in situ* hybridization (ref. 5; B. Harfe and A.F., unpublished observations). The *mex-3B* dsRNA produced 100% embryonic arrest, whereas >90% of embryos produced after the antisense injections hatched. Antisense probes for the *mex-3A* portion of *mex-3* were used to assay distribution of the endogenous *mex-3* mRNA (dark stain). four-cell-stage embryos are shown; similar results were observed from the one to eight cell stage and in the germ line of injected adults. **a**, Negative control showing lack of staining in the absence of the hybridization probe. **b**, Embryo from uninjected parent (showing normal pattern of endogenous *mex-3* RNA²⁰). **c**, Embryo from a parent injected with purified *mex-3B* antisense RNA. These embryos (and the parent animals) retain the *mex-3* mRNA, although levels may be somewhat less than wild type. **d**, Embryo from a parent injected with dsRNA corresponding to *mex-3B*; no *mex-3* RNA is detected. Each embryo is approximately 50 μ m in length.

cells. The phenotypes seen in *unc-54* and *hlh-1* null mutants, in particular, are known to result from many defective muscle cells^{11,16}. To examine interference effects of dsRNA at a cellular level, we used a transgenic line expressing two different green fluorescent protein (GFP)-derived fluorescent-reporter proteins in body muscle. Injection of dsRNA directed to *gfp* produced marked decreases in the fraction of fluorescent cells (Fig. 2). Both reporter proteins were absent from the affected cells, whereas the few cells that were fluorescent generally expressed both GFP proteins.

The mosaic pattern observed in the *gfp*-interference experiments was nonrandom. At low doses of dsRNA, we saw frequent interference in the embryonically derived muscle cells that are present when the animal hatches. The interference effect in these differentiated cells persisted throughout larval growth: these cells produced little or no additional GFP as the affected animals grew. The 14 postembryonically derived striated muscles are born during early larval stages and these were more resistant to interference. These cells have come through additional divisions (13–14 divisions versus 8–9 divisions for embryonic muscles^{18,19}). At high concentrations of *gfp* dsRNA, we saw interference in virtually all striated body-wall muscles, with occasional lone escaping cells, including cells born during both embryonic and postembryonic development. The non-striated vulval muscles, which are born during late larval development, appeared to be resistant to interference at all tested concentrations of injected dsRNA.

We do not yet know the mechanism of RNA-mediated interference in *C. elegans*. Some observations, however, add to the debate about possible targets and mechanisms.

First, dsRNA segments corresponding to various intron and promoter sequences did not produce detectable interference (Table 1). Although consistent with interference at a post-transcriptional level, these experiments do not rule out interference at the level of the gene.

Second, we found that injection of dsRNA produces a pronounced decrease or elimination of the endogenous mRNA transcript (Fig. 3). For this experiment, we used a target transcript (*mex-3*) that is abundant in the gonad and early embryos²⁰, in which straightforward *in situ* hybridization can be performed². No endogenous *mex-3* mRNA was observed in animals injected with a dsRNA segment derived from *mex-3*. In contrast, animals into which purified *mex-3* antisense RNA was injected retained substantial endogenous mRNA levels (Fig. 3d).

Third, dsRNA-mediated interference showed a surprising ability to cross cellular boundaries. Injection of dsRNA (for *unc-22*, *gfp* or *lacZ*) into the body cavity of the head or tail produced a specific and robust interference with gene expression in the progeny brood (Table 2). Interference was seen in the progeny of both gonad arms, ruling out the occurrence of a transient 'nicking' of the gonad

Table 2 Effect of site of injection on interference in injected animals and their progeny

dsRNA	Site of injection	Injected-animal phenotype	Progeny phenotype
None	Gonad or body cavity	No twitching	No twitching
None	Gonad or body cavity	Strong nuclear and mitochondrial GFP expression	Strong nuclear and mitochondrial GFP expression
<i>unc22B</i>	Gonad	Weak twitchers	Strong twitchers
<i>unc22B</i>	Body-cavity head	Weak twitchers	Strong twitchers
<i>unc22B</i>	Body-cavity tail	Weak twitchers	Strong twitchers
<i>gfpG</i>	Gonad	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
<i>gfpG</i>	Body-cavity tail	Lower nuclear and mitochondrial GFP expression	Rare or absent nuclear and mitochondrial GFP expression
<i>lacZL</i>	Gonad	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression
<i>lacZL</i>	Body-cavity tail	Lower nuclear GFP expression	Rare or absent nuclear-GFP expression

The GFP-reporter strain PD4251, which expresses both mitochondrial GFP and nuclear GFP-LacZ, was used for injections. The use of this strain allowed simultaneous assay for interference with *gfp* (fainter overall fluorescence), *lacZ* (loss of nuclear fluorescence) and *unc-22* (twitching). Body-cavity injections into the tail region were carried out to minimize accidental injection of the gonad; equivalent results have been observed with injections into the anterior body cavity. An equivalent set of injections was also performed into a single gonad arm. The entire progeny broods showed phenotypes identical to those described in Table 1. This included progeny of both injected and uninjected gonad arms. Injected animals were scored three days after recovery and showed somewhat less dramatic phenotypes than their progeny. This could be partly due to the persistence of products already present in the injected adult. After injection of double-stranded *unc22B*, a fraction of the injected animals twitch weakly under standard growth conditions (10 out of 21 animals). Levamisole treatment led to twitching of 100% (21 out of 21) of these animals. Similar effects (not shown) were seen with double-stranded *unc22A*. Injections of double-stranded *gfpG* or double-stranded *lacZL* produced a dramatic decrease (but not elimination) of the corresponding GFP reporters. In some cases, isolated cells or parts of animals retained strong GFP activity. These were most frequently seen in the anterior region and around the vulva. Injections of double-stranded *gfpG* and double-stranded *lacZL* produced no twitching, whereas injections of double-stranded *unc22A* produced no change in the GFP-fluorescence pattern.

in these injections. dsRNA injected into the body cavity or gonad of young adults also produced gene-specific interference in somatic tissues of the injected animal (Table 2).

The use of dsRNA injection adds to the tools available for studying gene function in *C. elegans*. In particular, it should now be possible functionally to analyse many interesting coding regions²¹ for which no specific function has been defined. Although the effects of dsRNA-mediated interference are potent and specific we have observed several limitations that should be taken into account when designing RNA-interference-based experiments. First, a sequence shared between several closely related genes may interfere with several members of the gene family. Second, it is likely that a low level of expression will resist RNA-mediated interference for some or all genes, and that a small number of cells will likewise escape these effects.

Genetic tools are available for only a few organisms. Double-stranded RNA could conceivably mediate interference more generally in other nematodes, in other invertebrates, and, potentially, in vertebrates. RNA interference might also operate in plants: several studies have suggested that inverted-repeat structures or characteristics of dsRNA viruses are involved in transgene-dependent co-suppression in plants^{22,23}.

There are several possible mechanisms for RNA interference in *C. elegans*. A simple antisense model is not likely: annealing between a few injected RNA molecules and excess endogenous transcripts would not be expected to yield observable phenotypes. RNA-targeted processes cannot, however, be ruled out, as they could include a catalytic component. Alternatively, direct RNA-mediated interference at the level of chromatin structure or transcription could be involved. Interactions between RNA and the genome, combined with propagation of changes along chromatin, have been proposed in mammalian X-chromosome inactivation and plant-gene co-suppression^{22,24}. If RNA interference in *C. elegans* works by such a mechanism, it would be new in targeting regions of the template that are present in the final mRNA (as we observed no phenotypic interference using intron or promoter sequences). Whatever their target, the mechanisms underlying RNA interference probably exist for a biological purpose. Genetic interference by dsRNA could be used by the organism for physiological gene silencing. Likewise, the ability of dsRNA to work at a distance from the site of injection, and particularly to move into both germline and muscle cells, suggests that there is an effective RNA-transport mechanism in *C. elegans*. □

Methods

RNA synthesis and microinjection. RNA was synthesized from phagemid clones by using T3 and T7 polymerase⁶. Templates were then removed with two sequential DNase treatments. When sense-, antisense-, and mixed-RNA populations were to be compared, RNAs were further purified by electrophoresis on low-gelling-temperature agarose. Gel-purified products appeared to lack many of the minor bands seen in the original 'sense' and 'antisense' preparations. Nonetheless, RNA species comprising <10% of purified RNA preparations would not have been observed. Without gel purification, the 'sense' and 'antisense' preparations produced notable interference. This interference activity was reduced or eliminated upon gel purification. In contrast, sense-plus-antisense mixtures of gel-purified and non-gel-purified RNA preparations produced identical effects.

Sense/antisense annealing was carried out in injection buffer (ref. 27) at 37 °C for 10–30 min. Formation of predominantly double-stranded material was confirmed by testing migration on a standard (nondenaturing) agarose gel: for each RNA pair, gel mobility was shifted to that expected for dsRNA of the appropriate length. Co-incubation of the two strands in a lower-salt buffer (5 mM Tris-Cl, pH 7.5, 0.5 mM EDTA) was insufficient for visible formation of dsRNA *in vitro*. Non-annealed sense-plus-antisense RNAs for *unc22B* and *gfpG* were tested for RNA interference and found to be much more active than the individual single strands, but twofold to fourfold less active than equivalent preannealed preparations.

After preannealing of the single strands for *unc22A*, the single electrophoretic species, corresponding in size to that expected for the dsRNA, was purified using two rounds of gel electrophoresis. This material retained a high degree of interference activity.

Except where noted, injection mixes were constructed so that animals would receive an average of 0.5×10^6 to 1.0×10^6 RNA molecules. For comparisons of sense, antisense, and double-stranded RNA activity, equal masses of RNA were injected (that is, dsRNA was used at half the molar concentration of the single strands). Numbers of molecules injected per adult are approximate and based on the concentration of RNA in the injected material (estimated from ethidium bromide staining) and the volume of injected material (estimated from visible displacement at the site of injection). It is likely that this volume will vary several-fold between individual animals; this variability would not affect any of the conclusions drawn from this work.

Analysis of phenotypes. Interference with endogenous genes was generally assayed in a wild-type genetic background (N2). Features analysed included movement, feeding, hatching, body shape, sexual identity, and fertility. Interference with *gfp* (ref. 25) and *lacZ* activity was assessed using *C. elegans* strain PD4251. This strain is a stable transgenic strain containing an integrated array (ccls4251) made up of three plasmids: pSAK4 (*myo-3* promoter driving mitochondrially targeted GFP); pSAK2 (*myo-3* promoter driving a nuclear-targeted GFP–LacZ fusion); and a *dpy-20* subclone²⁶ as a selectable marker. This strain produces GFP in all body muscles, with a combination of mitochondrial and nuclear localization. The two distinct compartments are easily distinguished in these cells, allowing easy distinction between cells expressing both, either, or neither of the original GFP constructs.

Gonadal injection was done as described²⁷. Body-cavity injections followed a similar procedure, with needle insertion into regions of the head and tail beyond the positions of the two gonad arms. Injection into the cytoplasm of intestinal cells is also effective, and may be the least disruptive to the animal. After recovery and transfer to standard solid media, injected animals were transferred to fresh culture plates at 16-h intervals. This yields a series of semisynchronous cohorts in which it was straightforward to identify phenotypic differences. A characteristic temporal pattern of phenotypic severity is observed among progeny. First, there is a short 'clearance' interval in which unaffected progeny are produced. These include impermeable fertilized eggs present at the time of injection. Second, after the clearance period, individuals that show the interference phenotype are produced. Third, after injected animals have produced eggs for several days, gonads can in some cases 'revert' to produce incompletely affected or phenotypically normal progeny.

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Correspondence and requests for materials should be addressed to A.F. (e-mail: fire@mail1.ciweb.edu).

Role of the histone deacetylase complex in acute promyelocytic leukaemia

Richard J. Lin^{*,†,‡}, Laszlo Nagy^{*†}, Satoshi Inoue[†], Wenlin Shao[§], Wilson H. Miller Jr[§] & Ronald M. Evans^{*†}

^{*}Howard Hughes Medical Institute, and [†]The Salk Institute for Biological Studies, La Jolla, California 92037, USA

[‡]Graduate Program in Molecular Pathology, University of California San Diego, School of Medicine, La Jolla, California 92093, USA

[§]Lady Davis Institute for Medical Research, McGill University Departments of Oncology and Medicine, Montreal, Quebec, Canada H3T 1E2

Non-liganded retinoic acid receptors (RARs) repress transcription of target genes by recruiting the histone deacetylase complex^{1–3} through a class of silencing mediators termed SMRT or N-CoR^{4,5}. Mutant forms of RAR α , created by chromosomal translocations with either the PML (for promyelocytic leukaemia)^{6–8} or the PLZF (for promyelocytic leukaemia zinc finger)^{9,10} locus, are oncogenic and result in human acute promyelocytic leukaemia (APL). PML–RAR α APL patients achieve complete remission following treatments with pharmacological doses of retinoic acids (RA); in contrast, PLZF–RAR α patients respond very poorly, if at all¹¹. Here we report that the association of these two chimaeric receptors with the histone deacetylase (HDAC) complex helps to determine both the development of APL and the ability of patients to respond to retinoids. Consistent with these observations, inhibitors of histone deacetylase dramatically potentiate retinoid-induced differentiation of RA-sensitive, and restore retinoid responses of RA-resistant, APL cell lines. Our findings suggest that oncogenic RARs mediate leukaemogenesis through aberrant chromatin acetylation, and that pharmacological manipulation of nuclear receptor co-factors may be a useful approach in the treatment of human disease.

Because both PML–RAR α and PLZF–RAR α inhibit normal retinoid signalling^{6–14}, we reasoned that identification of factors associated with these proteins might provide mechanistic insights into their oncogenic functions. PLZF–RAR α retains the autonomous repression domain, the BTB/POZ (for bric-à-brac/tramtrack/broad complex, poxvirus and zinc-finger) domain, from PLZF¹⁵. Because deletion of this domain abolishes the biological functions of PLZF–RAR α *in vivo*^{16,17}, we investigated whether it might associate directly with components of the nuclear receptor co-repressor complex^{1–3}. By using an *in vitro* interaction assay, we found that radiolabelled full-length mSin3A and histone deacetylase 1 (HDAC1), but not mSin3B, were specifically retained on matrix-

bound fusion proteins of glutathione S-transferase with the BTB/POZ domain of PLZF (GST–PLZF; Fig. 1a). Results from a yeast two-hybrid assay showed that PLZF interacts with all known components of the co-repressor complex *in vivo* (Fig. 1b). We further mapped the PLZF interaction domain in mSin3A to the paired amphipathic helix 1 (PAH1, residues 112–192) by a mammalian two-hybrid assay (Fig. 1c). Finally, using a co-immunoprecipitation assay from nuclear extracts of transfected CV1 cells, we confirmed that PLZF, SMRT, mSin3A and HDAC1 form a complex in mammalian cells (Fig. 1d). These results, together with the finding that SMRT interacts with another BTB/POZ oncoprotein, LAZ3/BCL6 (ref. 18), demonstrated that this family of transcriptional factors recruits histone deacetylases to repress transcription and implicates histone deacetylases in cellular transformation.

By using a yeast two-hybrid assay, we demonstrated that PLZF–RAR α interacts directly with both SMRT and mSin3A, whereas PML–RAR α interacts only with SMRT¹⁹ (Fig. 2B). Most importantly, we showed using a co-immunoprecipitation assay that HDAC1 exists in a complex with either PLZF–RAR α or PML–RAR α in transfected CV1 cells (Fig. 2C). Furthermore, a similar assay using nuclear extracts of the NB4 cells established from a patient with t(15;17) APL²⁰ indicated that endogenous HDAC1 can be co-precipitated with an anti-PML antibody (Fig. 2D). The presence of endogenous PML–RAR α was confirmed by immunoblotting analyses using anti-PML and anti-RAR α antibodies (data

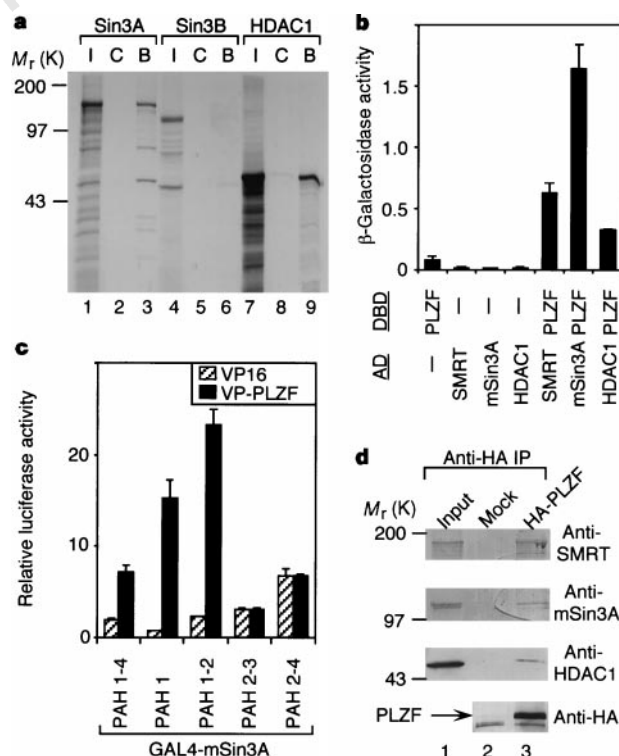


Figure 1 Association of co-repressors and HDAC1 with PLZF. **a**, SDS-PAGE analysis of ³⁵S-labelled mSin3A, mSin3B or HDAC1 proteins retained on immobilized GST–PLZF affinity matrices. I, 20% input; C, GST control; B, bound. **b**, Interactions between PLZF and full-length SMRT, mSin3A or HDAC1 in a yeast two-hybrid assay. **c**, Interactions between GAL4–DBD fusions of different PAHs of mSin3A and VP16 fusion of PLZF are analysed by a mammalian two-hybrid assay in CV1 cells. **d**, PLZF associates with the histone deacetylase complex *in vivo*. CV1 cells were transfected with either vectors only (mock) or plasmids encoding HA–PLZF, SMRT, mSin3A and HDAC1 (HA–PLZF) and nuclear extracts were immunoprecipitated (IP) using anti-HA antibodies followed by immunoblotting analyses using antibodies against SMRT, mSin3A and HDAC1. In lane 1, ~100 μ g of nuclear extract was applied to ascertain the positions of blotted proteins (input).

not shown). Because both PML-RAR α and PLZF-RAR α localize in microspeckle-like nuclear structures and/or perinuclear spaces for PML-RAR α ^{16,21,22}, we examined whether SMRT localizes to the same domains. Haemagglutinin (HA)-tagged PLZF-RAR α or PML-RAR α and SMRT were expressed in 293T cells and their subcellular localization determined by indirect immunofluorescence. Complete co-localization of SMRT with PLZF, PLZF-RAR α or PML-RAR α was observed (Fig. 2E, a–d, g, h). In contrast, staining of PML revealed that SMRT does not normally localize in PML oncogenic domains^{21,22} (Fig. 2E, e, f), consistent with previous findings that PML, in the absence of RAR α fusion, does not interact with SMRT^{4,19}. Taken together, our results strongly suggest that *in vivo* both chimaeric receptors associate with the histone deacetylase complex.

Despite the fact that both PML-RAR α and PLZF-RAR α APLs are clinically indistinguishable, they differ in their response to RA. PML-RAR α APL patients respond to pharmacological concentrations of RA and enter disease remission, whereas PLZF-RAR α patients are resistant¹¹. Because SMRT and the histone deacetylase complex dissociate from wild-type RARs on ligand binding^{3,4}, we investigated whether abnormal association of co-repressors and histone deacetylases with chimaeric receptors could explain the differences in RA sensitivity. We first performed an *in vitro* interaction assay, in which radiolabelled PLZF-RAR α , but not RAR α , was retained on the GST-HDAC1 affinity matrix (Fig. 3a). Surprisingly, addition of all-*trans* RA does not affect the binding of PLZF-RAR α (Fig. 3a). A similar ligand-insensitive interaction between PLZF-RAR α and mSin3A was also observed (data not shown). Because the RA-binding affinity of PLZF-RAR α is similar to that of wild-type RAR α ^{16,17}, we concluded that the fusion receptor constitutively associates with co-repressors and histone deacetylases through the PLZF moiety. We then examined the ligand-dependent

dissociation of SMRT from PML-RAR α using a gel mobility-shift assay because PML-RAR α forms stable homodimers on DNA *in vivo*^{13,23}. This analysis indicated that all-*trans* RA can dissociate SMRT from both RAR α and PML-RAR α complexes, but that substantially higher concentrations are required for PML-RAR α (Fig. 3b). This reduced RA sensitivity was also observed in a mammalian two-hybrid assay which showed a difference of two orders of magnitude in the effector concentration for half-maximal response (EC₅₀) between PML-RAR α (~1 μ M) and RAR α (~5 nM; data not shown). These results provide an explanation for the previous finding that PML-RAR α -expressing haematopoietic cells require high doses (>10⁻⁷ M) of RA to differentiate^{4,24}, and suggest that a more tightly associated PML-RAR α /SMRT complex may shift the physiological RA dose response towards the pharmacological range *in vivo* and correspondingly decrease the retinoid sensitivities of myeloid precursors. The striking correlation of the co-repressor dissociation from fusion receptors *in vitro* with distinct RA sensitivities of APL patients prompted us to examine further the interaction between SMRT and PML-RAR α in RA-resistant cases of APL. A mutation in PML-RAR α that impairs ligand binding (PML-RAR α , m4) has been identified in a RA-resistant subclone of NB4 cells (NB4-R4)²⁵. By using a gel-shift assay we found that all-*trans* RA could not release SMRT from this PML-RAR α mutant (Fig. 3c) suggesting that the phenotypic RA resistance may couple directly to the inability to dissociate the co-repressor complex and activate retinoid target genes.

Both PML-RAR α and PLZF-RAR α inhibit terminal differentiation of haematopoietic cell lines *in vitro*^{14,17,24} and block myeloid maturation *in vivo*²⁶. On the basis of the above results, we hypothesized that the common differentiation block by PML-RAR α and PLZF-RAR α is due to the association of the co-repressor complex and the inability to dissociate this complex using physiological

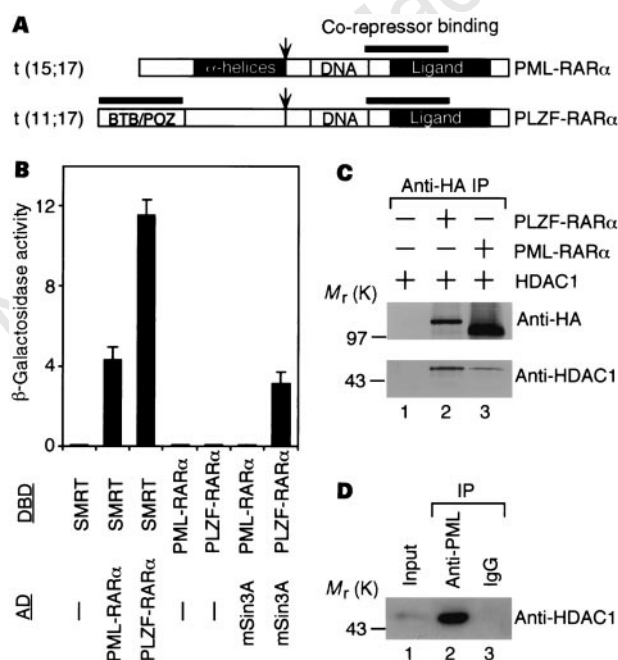
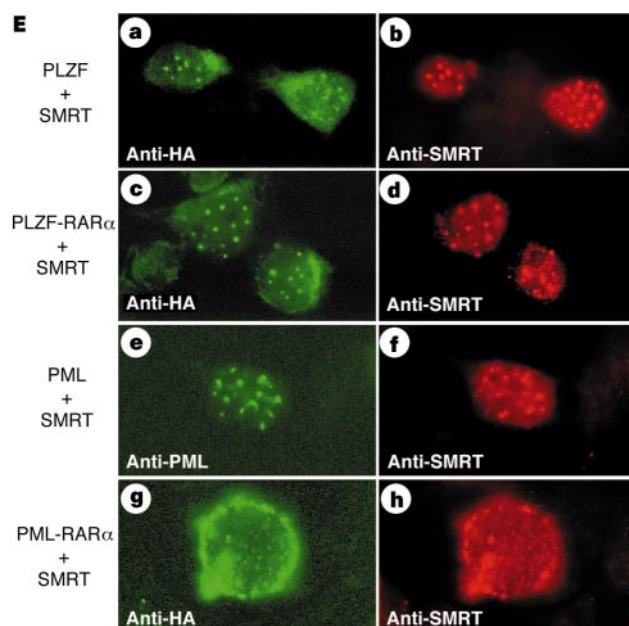


Figure 2 Association of co-repressors and histone deacetylases with PLZF-RAR α and PML-RAR α . **A**, Schematic representation of chromosomal translocations that generate PLZF-RAR α and PML-RAR α chimaeras. Co-repressor binding sites are indicated and arrows show translocation break points. **B**, Interactions between SMRT and mSin3A with full-length PML-RAR α and PLZF-RAR α in a yeast two-hybrid assay. **C**, HDAC1 associates with both fusion proteins (PML-RAR α and PLZF-RAR α) in transfected CV1 cells. Immunoblotting analyses of anti-HA immunoprecipitates from nuclear extracts of CV1 cells transfected with HDAC1 and HA-tagged PLZF-RAR α or PML-RAR α . **D**, Endogenous HDAC1 and



PML-RAR α are associated. Nuclear extracts from NB4 cells (2mg) were immunoprecipitated (IP) with either anti-PML or nonspecific IgG and blotted with anti-HDAC1. In lane 1, ~100 μ g of nuclear extract was applied to ascertain position of HDAC1 (Input). **E**, SMRT co-localizes with both chimaeric receptors in nuclear microspeckles. Immunofluorescence of 293T cells transfected with indicated plasmids and detected by anti-HA (**a**, **c** and **g**), anti-PML (**e**) and anti-SMRT (**b**, **d**, **f** and **h**). Typical PML oncogenic domains (PODs) are seen in **e**, and perinuclear staining of PML-RAR α is apparent in **g**.

concentrations of RA. We further suggested that the potential of APL patients to respond to RA is determined by the sensitivity of the interaction between the chimaeric receptor and the co-repressor complex to RA. The discovery of specific inhibitors of mammalian histone deacetylases²⁷ and the subsequent demonstration that these inhibitors block repression mediated by histone deacetylases^{2,3} allowed us to test these hypotheses by transient transfection assays. On UAS reporters, transfected GAL4-DBD fusions of PLZF-RAR α were poorly activated by all-*trans* RA compared with GAL4-RAR α (Fig. 4a, columns 1 and 2), presumably as a result of the constitutive association of histone deacetylases. Remarkably, the histone deacetylase inhibitors Trichostatin A (TSA) or sodium butyrate could synergize with all-*trans* RA to activate reporters (Fig. 4a, columns 3 and 4), suggesting that the inhibition of histone deacetylase activity enables RA to activate

through the RAR α moiety. Moreover, under conditions where overexpression of PLZF-RAR α or PML-RAR α inhibits retinoid response on β -RARE reporters⁶⁻¹⁴, TSA was able to restore nearly full RA activation in the presence of either of the fusion proteins (Fig. 4b). These results led us to investigate whether TSA has any effect on RA-induced differentiation of NB4 cells. As monitored by the Nitroblue tetrazolium (NBT) reduction assay, we found that low concentrations of TSA (50 nM) dramatically enhanced the differentiation effect of all-*trans* RA but had no effect alone (Fig. 4c, top). This differentiation-enhancing effect is mediated through the RA signalling pathway because we observed a strong synergistic effect of TSA on RA induction of the endogenous retinoid target gene *CD18* (data not shown). Taken together, our results strongly suggest that transcriptional repression mediated by histone deacetylase is required for leukaemias induced by PML-RAR α and PLZF-RAR α , and that inhibition of this repression could potentially enhance the retinoid responses of APL patients.

Although PML-RAR α APL patients respond to pharmacological doses of RA and achieve complete remission, many of them relapse and acquire permanent retinoid resistance²⁸. We wished to determine whether TSA could overcome such resistance using NB4-R4 cells as a model. We found that high concentrations (200 nM) of TSA partially restored RA-induced differentiation, as measured by

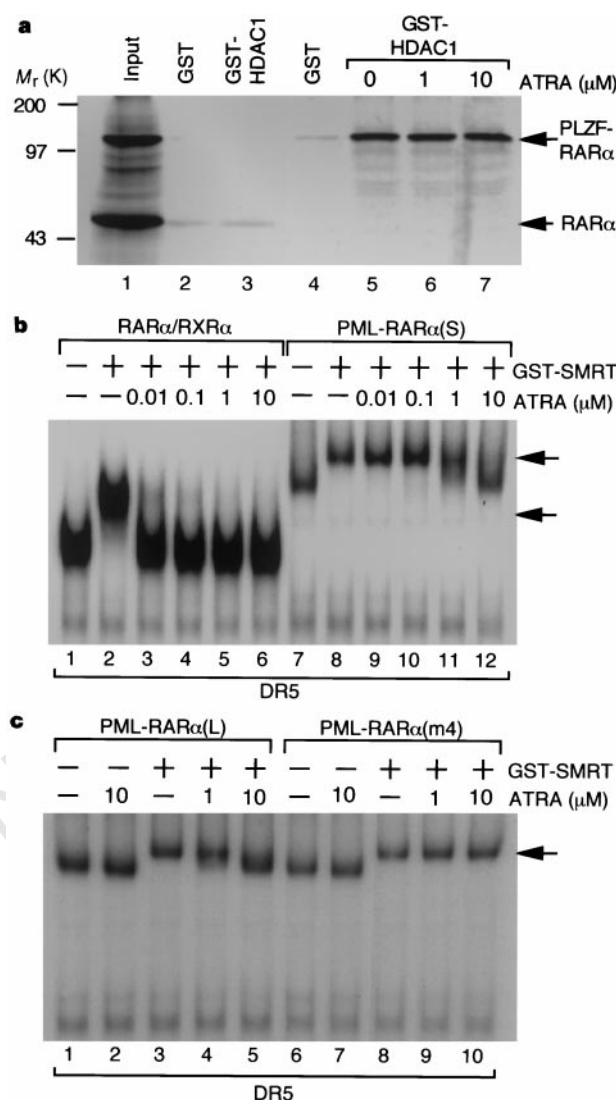


Figure 3 Impaired ligand-dependent dissociation of co-repressors from PLZF-RAR α and PML-RAR α proteins. **a**, The effect of indicated concentrations of all-*trans* RA (ATRA) on the interaction between PLZF-RAR α and HDAC1 analysed by an *in vitro* interaction assay using either GST or GST-HDAC1 as affinity matrices; 20% of the input is shown (lane 1). **b**, Gel-shift analysis of the interaction between GST-SMRT (receptor interaction domain) and RAR α /RXR α heterodimers or PML-RAR α (S denotes short isoform)⁶ homodimers on DR5 elements and the effect of ATRA. Positions of shifted complexes were indicated by arrows. **c**, Dissociation of GST-SMRT from either PML-RAR α (L denotes long isoform)⁶ or PML-RAR α (m4) homodimers on DR5 elements in the presence of indicated concentrations of ATRA. Arrows showed positions of shifted complexes.

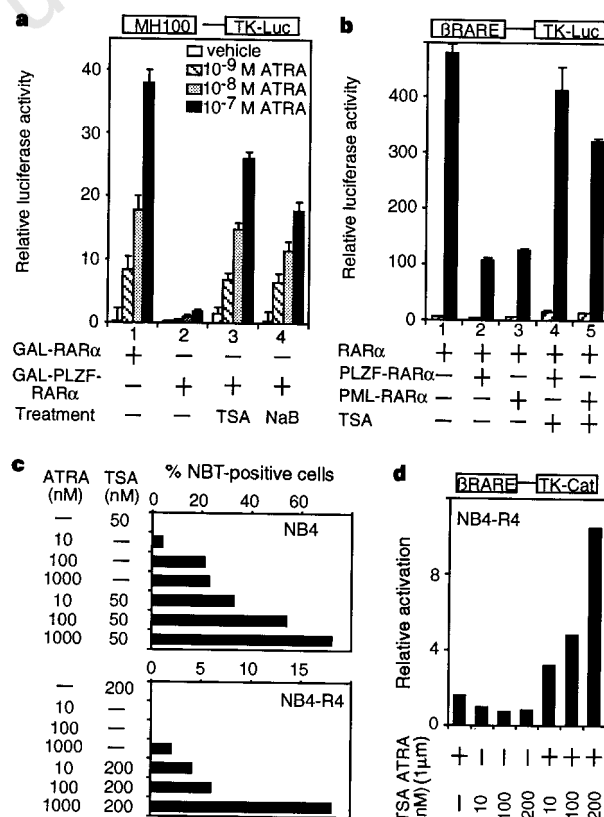


Figure 4 Histone deacetylase inhibitors suppress oncogenic activities of PLZF-RAR α and PML-RAR α . **a**, Histone deacetylase inhibitors TSA (150 nM) or sodium butyrate (NaB) (5 mM) synergized with all-*trans* RA (ATRA) to activate GAL-PLZF-RAR α on TK-MH100x4-Luc receptors in transfected CV1 cells. **b**, TSA (150 nM) overcame the dominant-negative inhibition of retinoid responses by PLZF-RAR α and PML-RAR α on TK- β RAREx2-Luc reporters in CV1 cells transfected with indicated plasmids. Filled and hatched bars indicated relative luciferase activities in the presence or absence of 100 nM ATRA, respectively. **c**, TSA potentiated differentiation effects of RA on NB4 cells (top) and NB4-R4 cells (bottom) as determined by the NBT assay. The y-axis indicates percentages of NBT-positive cells after treatments with ATRA and/or TSA for 3 days relative to control cells (no NBT-positive cells). **d**, TSA synergized with ATRA to activate TK- β RARE-CAT reporters in transfected NB4-R4 cells. Fold activation relative to control is shown.

significant increases in the number of NBT-positive cells (Fig. 4c, bottom). Although this is a surprising result considering the low RA-binding affinity of the PML-RAR α mutant in these cells, it is possible that endogenous wild-type RARs²⁵ could mediate at least part of the differentiation effects of RA. This speculation is supported by our finding that TSA restored RA signalling on β -RARE reporters in NB4-R4 cells in a dose-dependent manner (Fig. 4d). These results clearly indicate that inhibition of the dominant-negative activity of the PML-RAR α mutant increases net RA transcriptional responses in these resistant cells.

In summary, our findings lead to two major conclusions. First, the demonstration that targeted recruitment of the histone deacetylase complex by PML-RAR α and PLZF-RAR α underlies the pathogenesis and RA responsiveness of APL establishes the first direct link between nuclear receptor co-repressors and human cancer. Second, the identification of histone deacetylases as molecular targets of oncogenes and the effective growth suppression of RA-sensitive as well as RA-resistant leukaemic cells by inhibitors of histone deacetylases suggest that pharmacological manipulation of nuclear-receptor co-factors is a viable approach to the treatment of human leukaemias.

Note added in proof: In an accompanying paper²⁹, Grignani *et al.* report similar findings with the nuclear receptor co-repressor N-CoR.

Methods

Plasmids, antibodies and chemicals. Mammalian expression vectors for SMRT⁴, PML and PML-RAR α ⁶, PLZF and PLZF-RAR α ¹⁰ and PML-RAR α (m4)²⁵ are as described. Mammalian expression vectors for mSin3A, HDAC1 and HA-tagged PLZF and PLZF-RAR α were constructed using the polymerase chain reaction (PCR) and cloned into pCMX. Yeast two-hybrid plasmids were cloned into either pGBT9 or pGAD424 (Clontech). GST fusions of the BTB/POZ domain of PLZF (residues 7–118), the receptor interaction domain of SMRT (residues 1,073–1,168), and full-length HDAC1 were cloned into pGEX (Pharmacia). All constructs were verified by sequencing. Rabbit anti-SMRT polyclonal antibody was produced in a rabbit injected with purified GST-SMRT (residues 38–448) proteins. Anti-PML antibody has been described²¹. Anti-mSin3A and anti-HA antibody were from Santa Cruz Biotechnology and Boehringer Mannheim, respectively. Histone deacetylase inhibitors were sodium butyrate (CalBiochem) and TSA (Waco Pure Chemical Industries).

In vitro interaction assays and electrophoretic mobility-shift assays. GST fusion proteins were produced and purified from *Escherichia coli* DH5 α cells. For a typical binding assay, 0.5 μ g of recombinant proteins were incubated with 20 μ l of ³⁵S-labelled proteins in G buffer (20 mM Tris, pH 7.9, 150 mM KCl, 1 mM EDTA, 4 mM MgCl₂, 0.2% NP-40, 10% glycerol) at 4 °C with gentle rocking. After extensive washing, bound proteins were resolved by SDS-PAGE and visualized by autoradiography. For the mobility-shift assays, *in vitro* translated receptors and purified GST fusion proteins were incubated with 1 μ g poly(dI:dC) on ice in a 24- μ l reaction containing 100 mM KCl, 6% glycerol, 16.7 mM Tris, pH 7.5, 0.1% NP-40, 1 mM DTT and all-trans RA as indicated. ³²P-labelled double-strand DR5 (5'-TCGACTCaggtcACACgaggtcGAGTC-3') oligonucleotide probes (150,000 c.p.m.) were then added and incubation was continued for 30 min at room temperature. The protein-DNA complexes were resolved on a 4.5% native polyacrylamide gel and visualized by autoradiography.

Immunoprecipitation and immunohistochemistry. CV1 cells (3 $\times$ 10⁷) were transfected with 20 μ g of each indicated plasmid and collected in ice-cold 1 $\times$ PBS 36 h after transfection. Transfected CV1 cells or NB4 cells were lysed by mild sonication in IP buffer (1 $\times$ PBS, 10% glycerol, 1 mM NaF, 1 mM Na₃VO₄, 0.1% NP-40, 1 mM PMSF plus protease inhibitors). Immunoprecipitation was performed at 4 °C overnight followed by western blotting. Immunohistochemistry was done as previously described²¹.

Yeast methods and transient transfection assays. Yeast two-hybrid assays were performed as described³ and results are presented as the average β -galactosidase activity of three independent transformants ($\pm$ s.d.). CV1 cells were transfected as described³ and data are presented as relative luciferase activity ($\pm$ s.d.) of triplicate values obtained from a representative experiment

that was repeated independently at least three times. When indicated, transfected cells were treated for 24 h with all-trans RA and histone deacetylase inhibitors. NB4-R4 cells were transfected by electroporation (GIBCO BRL) and the chloramphenicol acetyltransferase assay was performed as described²⁵. Relative activation of reporters from one representative experiment is shown.

Cell culture and differentiation analysis. NB4 and NB4-R4 cells were cultured in RPMI medium supplemented with 10% fetal bovine serum. Differentiation of these cells by RA and/or TSA was monitored by the NBT assay performed essentially as described¹⁴.

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Correspondence and requests for materials should be addressed to R.M.E. (evans@salk.edu).

Fusion proteins of the retinoic acid receptor- α recruit histone deacetylase in promyelocytic leukaemia

Francesco Grignani^{*†}, Silvia De Matteis^{*}, Clara Nervi[‡], Lucia Tomassoni[§], Vania Gelmetti^{*}, Mario Ciocce[§], Mirco Fanelli[§], Marthin Ruthardt^{||}, Fabiana F. Ferrara[‡], Iris Zamir[§], Christian Seiser[#], Fausto Grignani^{*}, Mitchell A. Lazar[¶], Saverio Minucci[§] & Pier Giuseppe Pelicci[§]

^{*} Istituto di Medicina Interna e Scienze Oncologiche, Perugia University, 06100 Perugia, Italy

[†] Department of Haematology and Oncology, Istituto Superiore di Sanità, 00161 Rome, Italy

[‡] Dipartimento di Istologia ed Embriologia Medica, University of Rome "La Sapienza", 00161 Rome, Italy

[§] European Institute of Oncology, Department of Experimental Oncology, 20141 Milan, Italy

[¶] Division of Endocrinology, Diabetes and Metabolism, Department of Medicine and Department of Genetics, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104, USA

[#] Institute of Molecular Biology, University of Vienna, Vienna Biocenter, Vienna, Austria

^{||} Department of Haematology, Johann Wolfgang Goethe-University, Frankfurt, Germany

The transforming proteins of acute promyelocytic leukaemias (APL) are fusions of the promyelocytic leukaemia (PML) and the promyelocytic leukaemia zinc-finger (PLZF) proteins with retinoic acid receptor- α (RAR α)^{1,2}. These proteins retain the RAR α DNA- and retinoic acid (RA)-binding domains, and their ability to block haematopoietic differentiation depends on the RAR α DNA-binding domain³⁻⁶. Thus RA-target genes are downstream effectors^{7,8}. However, treatment with RA induces differentiation of leukaemic

blast cells and disease remission in PML-RAR α APLs, whereas PLZF-RAR α APLs are resistant to RA^{1,2}. Transcriptional regulation by RARs involves modifications of chromatin by histone deacetylases, which are recruited to RA-target genes by nuclear co-repressors^{9,10}. Here we show that both PML-RAR α and PLZF-RAR α fusion proteins recruit the nuclear co-repressor (N-CoR)-histone deacetylase complex through the RAR α CoR box. PLZF-RAR α contains a second, RA-resistant binding site in the PLZF amino-terminal region. High doses of RA release histone deacetylase activity from PML-RAR α , but not from PLZF-RAR α . Mutation of the N-CoR binding site abolishes the ability of PML-RAR α to block differentiation, whereas inhibition of histone deacetylase activity switches the transcriptional and biological effects of PLZF-RAR α from being an inhibitor to an activator of the RA signalling pathway. Therefore, recruitment of histone deacetylase is crucial to the transforming potential of APL fusion proteins, and the different effects of RA on the stability of the PML-RAR α and PLZF-RAR α co-repressor complexes determines the differential response of APLs to RA.

High levels of histone acetylation have been linked to transcriptionally active chromatin, and histone deacetylase activity is thought to lead to a repressive chromatin conformation^{11,12}. In the absence of ligand, RARs repress transcription by recruiting histone deacetylase through N-CoR and the related SMRT¹³⁻¹⁵. RA releases this interaction, favouring recruitment of coactivators and transcription¹³⁻¹⁵. As previously reported for SMRT¹⁶, PML-RAR α and PLZF-RAR α interact with N-CoR *in vitro* (Fig. 1a). To demonstrate their association *in vivo*, we performed co-immunoprecipitation experiments in the NB4 cell line (from a PML-RAR α APL) and U937 cells expressing PML-RAR α ¹⁷ or PLZF-RAR α ⁸. Anti-N-CoR blots of anti-RAR α , anti-PML or anti-PLZF immunoprecipitates revealed the existence of N-CoR complexes with PML-RAR α or PLZF-RAR α (Fig. 1b). We then analysed the same immunoprecipitates for histone deacetylase activity. Anti-PML and anti-RAR α antibodies specifically precipitated significant levels of histone deacetylase activity in extracts from NB4 and U937-PML/RAR α cells; similarly, anti-PLZF and anti-RAR α antibodies precipitated histone deacetylase activity from U937 PLZF-RAR α cells. Histone deacetylase activity was also detected in anti-haemagglutinin (HA) immunoprecipitates from U937 cells expressing HA-tagged PML-RAR α ¹⁸, confirming

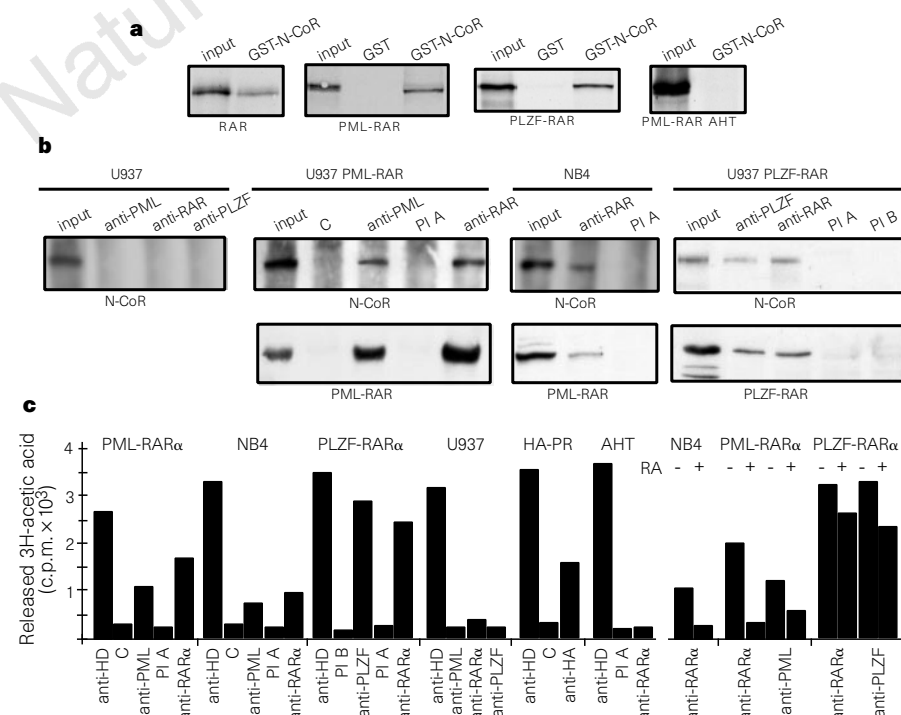


Figure 1 Interactions of PML-RAR α and PLZF-RAR α with N-CoR and histone deacetylase. **a**, *In vitro*-translated RAR α , PML-RAR α , PLZF-RAR α and PML-RAR α AHT (input) were precipitated with GST or the GST-N-CoR 1425-2453 protein. **b**, Immunoprecipitates with the anti-PML¹⁸, anti-RAR α and anti-PLZF⁸ antibodies were obtained from nuclear extracts of the indicated cells and blotted with anti-N-CoR or anti-RAR α antibodies, as indicated. PIA and PIB are anti-RAR α and anti-PLZF pre-immune sera; C, an unrelated monoclonal antibody (anti-CD14). **c**, Histone deacetylase (HD) activity of the indicated immunoprecipitates from: U937 PML-RAR α , NB4, U937 PLZF-RAR α , parental U937 (ref. 28), U937 expressing HA-tagged PML-RAR α (HA-PR), U937 expressing the PML-RAR α AHT mutant (AHT), NB4, PML-RAR α and PLZF-RAR α . RA, nuclear extracts treated with 20 μ M RA.

the specific association of the fusion protein with histone deacetylase activity (Fig. 1c).

To investigate the role of N-CoR in the recruitment of histone deacetylase by the fusion proteins, we introduced into the PML-RARα CoR box the triple point mutation that abolishes binding of RARs to N-CoR (PML-RARα AHT¹⁹). N-CoR failed to bind PML-RARα AHT *in vitro* (Fig. 1a), suggesting that the interaction between PML-RARα and N-CoR is mediated exclusively through the CoR box of RARα. Anti-RARα immunoprecipitates from U937 PML-RARα AHT cells did not have histone deacetylase activity (Fig. 1c), indicating that PML-RARα recruits histone deacetylase through N-CoR and that a complex of PML-RARα and N-CoR-histone deacetylase exists *in vivo*.

To explore the biological relevance of the interaction of PML-RARα with N-CoR-histone deacetylase, we compared the effects of PML-RARα and PML-RARα AHT on the differentiation of haematopoietic cells. Expression of PML-RARα blocked the differentiation of U937 cells by vitamin D3 and TGF-β as reported¹⁷, but PML-RARα AHT expression had no effect (Fig. 2a, b). Notably, PML-RARα and PML-RARα AHT showed an identical stimulatory activity on the RA-induced differentiation of U937 cells (Fig. 2c). N-CoR binding is therefore critical for the capacity of PML-RARα to block differentiation, but not to mediate the response to RA.

Cells expressing PLZF-RARα, but not PML-RARα, are resistant to RA, and the RA response is (for some genes) severely impaired⁸. A different regulation of the fusion protein N-CoR-histone deacetylase complexes by RA could underlie their different effects on RA-mediated transcription. We therefore investigated the association of PML-RARα, PLZF-RARα and RARα with N-CoR as a function of RA binding *in vitro*. The shape of the resulting association curves differed markedly (Fig. 3a). Low RA concentrations (10^{-9} to 10^{-7} M) did not affect the ability of PML-RARα to associate with N-CoR, but they induced a marked decrease (40–65%) in the amounts of RARα bound to N-CoR. The higher binding affinity of PML-RARα to N-CoR suggests that PML-RARα might act as a potent transcriptional repressor on RA target genes at low, near-physiological, concentrations of RA, and could explain the loss of transforming

potential by PML-RARα AHT. At high RA concentrations (1×10^{-8} to 2×10^{-5} M), PML-RARα (and RARα) were fully dissociated from N-CoR, thereby providing a mechanism for the RA-dependent release of the differentiation block induced by PML-RARα. Instead, a fraction (about 30%) of the complex of PLZF-RARα and N-CoR seems to be resistant to dissociation even at the highest concentrations of RA (Fig. 3a).

To further characterize the interaction between PLZF-RARα and N-CoR, we analysed the potential of N-CoR to bind PLZF-RARα carrying the AHT mutation. PLZF-RARα AHT retained the capacity of PLZF-RARα to interact with N-CoR, and this interaction was not affected by treatment with 10^{-5} M RA, suggesting that an additional region(s) of PLZF-RARα binds N-CoR (Fig. 3c). We found a direct interaction between PLZF and N-CoR (Fig. 3c). To map the N-CoR region(s) involved in complex formation with PLZF, we evaluated the potential of a variety of GST-N-CoR fusion proteins to interact with *in vitro*-translated PLZF (Fig. 3b). Results

Figure 2 Effects of PML-RARα AHT on differentiation. PML-RARα and PML-RARα AHT cDNAs were expressed in U937 cells using the Zn-inducible metallothionein promoter¹⁸. **a**, Anti-RARα western blotting from control, PML-RARα and PML-RARα AHT U937 cells displaying Zn-inducible expression. **b**, Effects of the fusion proteins on vitamin D3/TGFβ-induced differentiation, monitored by flow cytometry of the differentiation antigen CD14 (ref. 17). **c**, Effects on RA-induced differentiation (percentage of NBT-reducing cells)¹⁷. Results are given as mean values ± s.d. from three experiments.

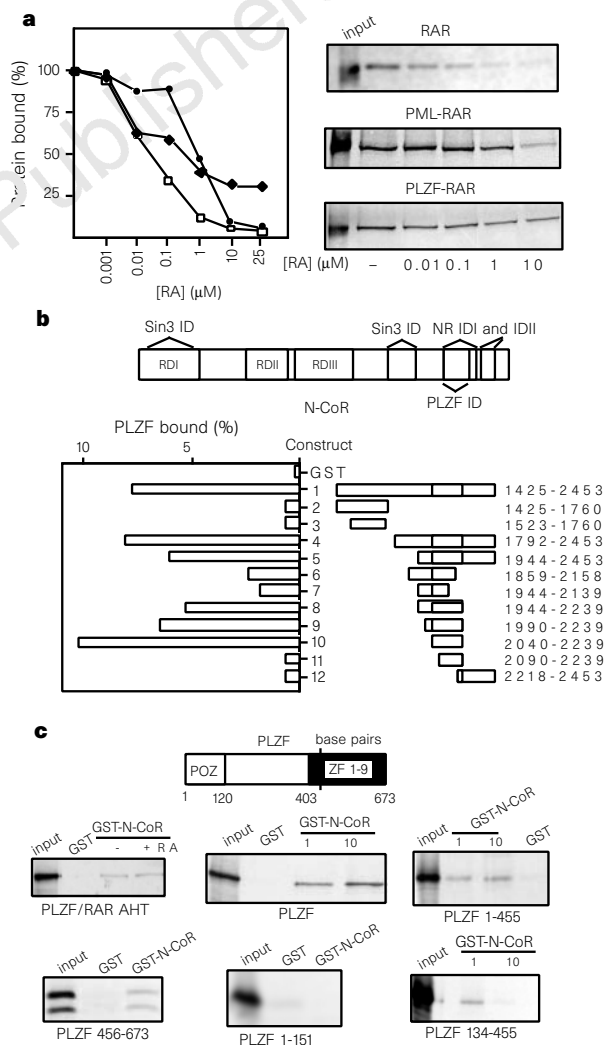
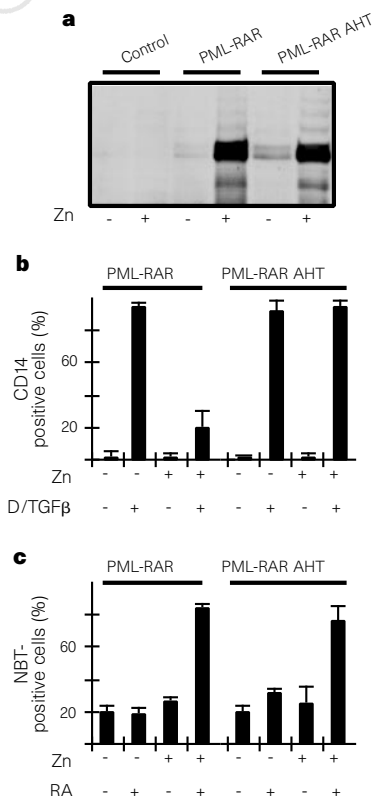


Figure 3 Differential N-CoR-fusion protein interactions. **a**, *In vitro* translated RARα (squares), PML-RARα (circles) and PLZF-RARα (diamonds) proteins were exposed to the indicated RA concentrations and precipitated with GST-N-CoR 1425-2453. Bound proteins (right panels are representative experiments) were quantified by phosphorimager and expressed as the percentage of proteins precipitated in the absence of RA. **b**, **c**, PLZF-N-CoR interaction mapping. **b**, Quantification of *in vitro* translated PLZF protein precipitated by GST-N-CoR proteins (boundaries and positions are shown). Results are expressed as the percentage of the amount of input PLZF protein. **c**, The indicated *in vitro* translated proteins (input) were precipitated with GST-N-CoR 1425-2543 (1), 2040-2239 (10) or GST proteins.

revealed that the integrity of amino acids 1944–2090 is critical for PLZF binding, and that residues 2040–2090 are essential. Because the efficiency of binding of constructs 6 and 7 (1859–2158) was markedly lower than construct 8 (1944–2239), region 2158–2239 also appears to be required for optimal N-CoR–PLZF binding. Mapping of the interacting region(s) in PLZF showed that both the 1–455 and the 456–673 PLZF polypeptides bound N-CoR. Region 1–455 (retained in PLZF–RAR α) includes the POZ domain, a protein–protein interaction domain involved in the binding of PLZF and BCL6 to SMRT^{16,20}. GST N-CoR amino acids 1425–2453 of GST–N-CoR failed to interact with the PLZF POZ domain, although it bound the PLZF 134–453 region, which is downstream

of the POZ domain. Amino acids 2040–2239 of GST–N-CoR (the minimal region of N-CoR which binds PLZF) failed to interact with either the POZ or the 134–453 region, suggesting that the POZ domain stabilizes the N-CoR–PLZF association.

In summary, PLZF–RAR α contains two N-CoR binding sites: the first is RA sensitive and maps within the CoR box of RAR α ; the second is RA resistant and maps to the N-terminal region of PLZF. Histone deacetylase activity was undetectable in RA-treated anti-RAR α or anti-PML immunoprecipitates from NB4 and U937 PML–RAR α cells. Strikingly, RA did not affect histone deacetylase activity in the anti-RAR α and anti-PLZF immunoprecipitates from U937 PLZF–RAR α cells (Fig. 1c). These results suggest that the presence of the RA-insensitive complexes of PLZF–RAR α and histone deacetylase at the promoters of RA target genes could be responsible for the negative effects of the fusion protein on RA-induced differentiation. We therefore investigated the effect of a specific inhibitor of histone deacetylase activity, trichostatin A (TSA)²¹, on RA-regulated expression of transglutaminase type II (TGase) and differentiation of U937 PLZF–RAR α cells. TGase expression is transcriptionally regulated by RA through the binding of RARs to a RA-responsive element in the gene promoter²². Transcriptional activation of TGase expression and differentiation mediated by RA are enhanced in PML–RAR α cells, compared with the parental U937 cells, but not in PLZF–RAR α cells²³ (Fig. 4). However, in these cells, in the presence of TSA, RA induced TGase activity (by a factor of fivefold) and differentiation (80–90% of CD14-positive cells) to the same levels as those observed in U937 PML–RAR α cells treated with RA (Fig. 4), thus establishing a link between the cellular levels of histone acetylation and the transcriptional and biological activity of PLZF–RAR α .

These data provide evidence for a biological function of the N-CoR–histone deacetylase complexes and the molecular mechanisms for the pathogenesis of APL and its treatment (Fig. 5). They also provide putative molecular targets and biological assays for new approaches to the treatment of this form of leukaemia. Given that a chromosomal translocation that fuses the product of the MOZ gene to the CBP histone acetyltransferase protein is associated with another form of acute myeloid leukaemia²⁴, deregulation of histone acetylation and altered chromatin organization might be an important molecular event in the processes leading to cell transformation in other neoplastic diseases.

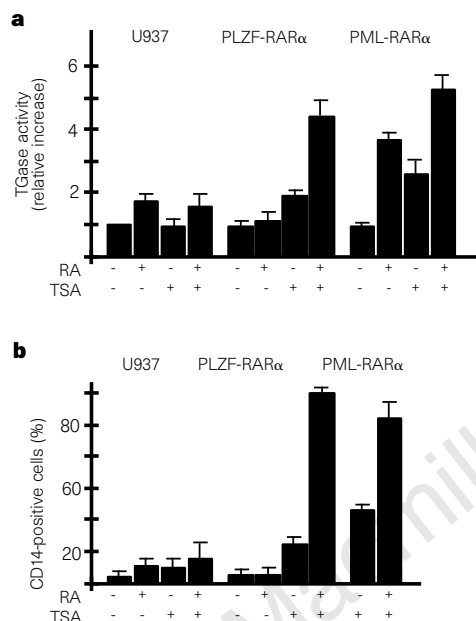


Figure 4 Effects of TSA on RA-induced TGase expression and differentiation of U937 PML–RAR α or PLZF–RAR α cells. The indicated cell lines were treated with TSA (120 nM) and/or RA (1 μ M), as indicated, and assayed for TGase activity²³ (a) or percentage CD14 expression (b) after 36 h. TGase activity is expressed as relative increase with respect to untreated cells.

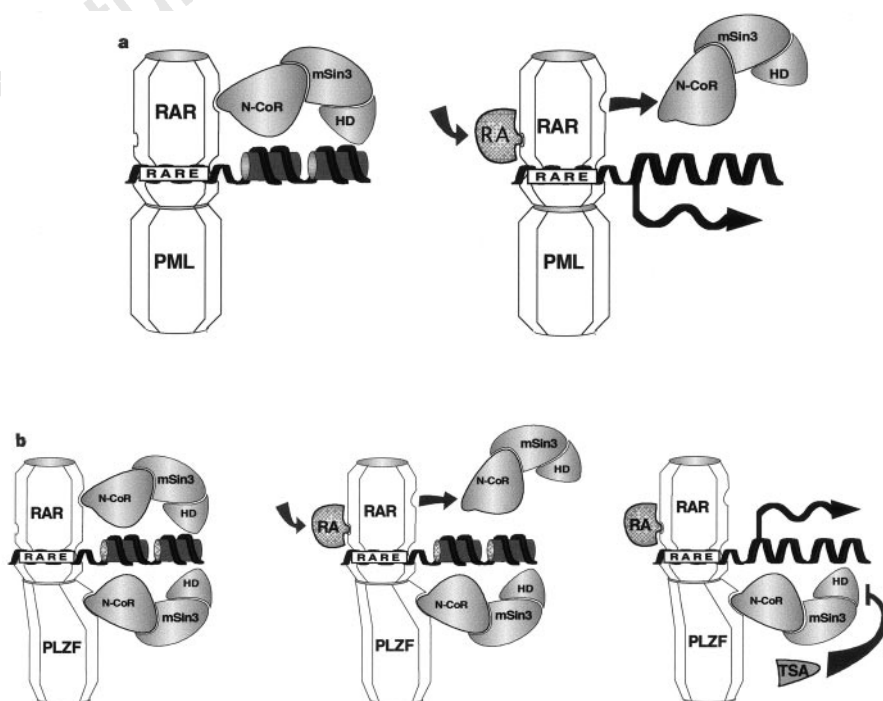


Figure 5 A model for the interactions of APL fusion proteins with the N-CoR–mSin3–histone deacetylase (HD) complex. **a**, DNA-bound PML–RAR α interacts with N-CoR (or SMRT) and recruits the mSin3–HD complex, decreasing histone acetylation and producing repressive chromatin organization and transcriptional repression. RA induces dissociation of the N-CoR–mSin3–HD complex, recruitment of coactivators with histone acetyltransferase activity (not shown), increased levels of histone acetylation, chromatin remodelling and transcriptional activation. **b**, PLZF–RAR α has two N-CoR binding sites that, even in the presence of RA, recruit the N-CoR–mSin3–HD complex and maintain transcription repression. TSA inhibits HD activity within the N-CoR complex, increasing histone acetylation, possibly through RA-recruited coactivators, and leading to transcription activation.

Note added in proof: Similar conclusions were reached by Lin *et al.*²⁹ studying the association of APL fusion proteins and SMRT. □

Methods

Mutants, constructs and cell lines. PML-RAR α and PLZF-RAR α AHT mutants¹⁹ were generated by the polymerase chain reaction (PCR) and subcloned in the pGMT-SV-NEO and pCDNA3 eukaryotic expression vectors. Stable U937 transfectants of PML-RAR α , PML-RAR α AHT and PLZF-RAR α cloned into pGMT-SV-NEO were obtained by electroporation. The used PLZF mutants were generated by PCR and cloned within the pCDNA3 vector.

In vitro binding assays with GST fusion proteins. The GST-N-CoR constructs 4–12 have been described previously²⁵. Constructs 1–3 were generated by PCR and cloned in the pGex4T-1 vector. GST beads containing the fusion proteins (10 μ g) were incubated in E1A buffer (50 mM HEPES, pH 7.8, 50 mM NaCl, 5 mM EDTA, 1 mM DTT, 0.1% NP40) with 5 μ l of *in vitro*-translated polypeptides. After washing, bound proteins were eluted by boiling in SDS-PAGE loading buffer, resolved by electrophoresis, and visualized by autoradiography.

Co-immunoprecipitation experiments and western blotting analysis. Nuclear extracts were prepared as described²⁶. Where indicated, RA (20 μ M) was added 1 h before incubation of the nuclear extracts with the appropriate antibodies or controls. Immunocomplexes were recovered by protein A-Agarose beads and resolved by electrophoresis. Anti-N-CoR antiserum was raised against the GST-N-CoR 1–224.

Histone deacetylase assay. Assays for histone deacetylase activity were performed as described²⁷. Immunoprecipitated complexes on protein A-agarose beads were incubated at 30 °C for 1 h with [³H]acetate-labelled chicken erythrocyte histones. The reaction was stopped by addition of 0.7-vol of 1M HCl–0.4 M acetate. Released [³H] acetic acid was extracted with ethyl acetate and quantified by liquid scintillation analysis. As a positive control, we measured histone deacetylase activity in anti-histone deacetylase-1 immunocomplexes.

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Correspondence and requests for materials should be addressed to P.G.P. (e-mail: pggellicci@ieo.cilea.it) and S.M. (sminucci@ieo.cilea.it).

correction

The cerebellar leucine-rich acidic nuclear protein interacts with ataxin-1

Antoni Matilla, Beena T. Koshy, Christopher J. Cummings, Toshiaki Isobe, Harry T. Orr & Huda Y. Zoghbi

Nature **389**, 974–978 (1997)

At the time of submission of this Letter, it escaped our attention that murine and human LANP sequences had previously been reported under different names in the following papers:

- Vaesen, M. *et al.* Purification and characterization of two putative HLA class II associated proteins: PHAPI and PHAPIL. *Biol. Chem. Hoppe-Seyler* **375**, 113–126 (1994).
- Li, M., Makkinje, A. & Damuni, Z. Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry* **35**, 6998–7002 (1996).
- Chen, T.-H. *et al.* Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. *Mol. Biol. Cell.* **7**, 2045–2056 (1996).
- Ulitzur, N., Humbert, M. & Pfeffer, S. R. Mapmodulin: A possible modulator of the interaction of microtubule-associated proteins with microtubules. *Proc. Natl Acad. Sci. USA* **94**, 5084–5089 (1997).

Although the gene products reported in these papers were isolated from peripheral tissues, the levels of LANP in cerebellar RNA are significantly higher than in peripheral tissues. Hence the central conclusion of our paper is unchanged. □

erratum

Fear conditioning induces associative long-term potentiation in the amygdala

Michael T. Rogan, Ursula V. Stäubli & Joseph E. LeDoux

Nature **390**, 604–607 (1997)

In Fig. 1a of this Letter, the labelling for the control rat (bottom panel) was erroneously relettered as 'Unpaired/Unpaired/Unpaired', whereas it should have read 'Unpaired/Unpaired/Unpaired'.

Also, the report of raw (not normalized) values of slope and amplitudes before training (third paragraph) should read 'slope: conditioned group, $-1.649 \pm 0.425 \mu\text{V ms}^{-1}$, control group, $-2.329 \pm 0.346 \mu\text{V ms}^{-1}$; *t*-test, $P > 0.05$; amplitude: conditioned group, $14.186 \pm 4.103 \mu\text{V}$, control group, $18.116 \pm 4.214 \mu\text{V}$; *t*-test, $P > 0.05$ '. Finally, on the third line of page 606, $P < 0.01$ (not > 0.01 as published). □

Light in the gloom for cell biologists

Opportunities abound throughout the world for young people interested in cell biology. The most successful will be those who can learn new skills and work collaboratively. But Europe needs to learn an urgent lesson from the United States if there is to be any future for biomedical research in the next millennium.

Brendan Horton

There should be more faculty positions for biomedical PhDs in US academic institutions over the next ten years: research funding from the National Institutes of Health (NIH) has outstripped inflation over the past decade and is likely to improve significantly in the next, and there are more jobs in industry. That was the message in last year's graduate education consensus conference report, published by the Federation of American Societies for Experimental Biology (FASEB), giving gloomy graduate students and postdocs grounds for optimism.

Pessimism about research careers may have resulted from unrealistic expectations and a lack of timely information about available employment (see table under CPST). After all, FASEB now reports that the number of tenure-track and tenured academic positions in the United States has remained fairly constant since 1981. During this time, the number of biomedical PhDs awarded increased from 3,845 in 1981 to 5,878 in 1995, dramatically increasing the competition for available positions.

The low turnover of faculty positions means that people stay longer as postdocs: in 1981, 23.6% of employed biomedical PhDs were on postdoctoral fellowships four years after receiving their degrees, compared with 32.1% in 1995. But the percentage of postdocs employed in research in industry has risen, and unemployment among biomedical science PhDs who are US citizens has remained remarkably low at 1.0-1.9% between 1973 and 1995. Improved reporting of career data should help keep expectations among career researchers more realistic.

Randy Schekman, chairman of the department of molecular and cell biology at the University of California, Berkeley, says: "There's a lot of gloom that the media spreads about prospects for academic positions and funding of junior faculty. In fact, things are better now than when I started over 20 years ago." He says recent reports have "contributed to the bad feelings" among young biomedical scientists. "When I started in the mid-1970s, the competition for the key positions was already pretty keen."

Schekman feels that news about federal funding is encouraging. The NIH budget has more than kept pace with inflation during the past ten years and many feel it will double in the next ten. Schekman says the young faculty members hired at Berkeley and other first-class institutions are often as successful



Schekman: sees encouraging signs.

as senior researchers at securing funds from the NIH. (This is not necessarily true in Europe, see p. 821.) And junior faculty are now typically offered set-up packages, often exceeding \$0.25 million. Before 1985, these funds were almost non-existent. "I had no set-up package when I started at Berkeley in 1976," says Schekman. Once a successful application has been made to the NIH, grants can also be obtained from private foundations, such as Pew, Searle, Packard, McKnight or Burroughs Wellcome. There are almost a dozen private organizations that give grants to junior faculty to bridge the postdoctoral and early faculty years. "The American Cancer Society, a major player among private foundations, has gone to providing competitive grants only to untenured faculty," he says.

For young researchers not in this select group, there are other opportunities, including careers in biotechnology, research in the government sector, patents work, investment consulting and teaching.

Industry has created many opportunities for PhDs in the United States. According to

the FASEB report, the percentage of biomedical PhDs employed in industry more than doubled between 1981 and 1995, to almost 32%. Schekman says these are often attractive positions, offering several years of freedom from looking for funds, involving research similar to that done in universities. "A lot of students see that chasing money, teaching and doing a lot of administration is not what they want to do," he says.

On the trail of scientists interested in doing research outside the academic sphere is Ken Johnson, a staffing consultant for Hoechst Marion Roussel. He is looking for bachelors, masters and PhD graduate researchers to fill scores of positions (see table), as the company has built a US\$70 million research centre on its 110-acre campus in Bridgewater, New Jersey, scheduled to open this month. These days, Johnson has to move quickly. "Companies no longer have the luxury of interviewing 20 candidates over six months." Competition for talent is fierce and good candidates typically find a job within six or eight weeks.

Doing a postdoc in industry can be a way for young researchers to test the water in this sector. David Bowen, currently a senior scientist with NeuralStem Biopharmaceuticals of College Park, Maryland, was nearing the

Developing 'chemical genetics'

The idea of an institute where synthetic chemists and biologists work together on common problems has for some time been a dream of Stuart Schreiber, of the department of chemistry and chemical biology at Harvard Medical School (HMS). That dream will be realized later this year as a result of an initiative involving Schreiber, Dan Tosteson, the outgoing dean of HMS, and Marc Kirschner (also at HMS).

The mission of the Harvard Institute of Chemistry and Cell Biology (ICCB) will be to understand protein function *in vivo* by using ligands that inhibit and/or activate proteins at controlled times. The co-directors are Schreiber and Timothy J. Mitchison from the department of cell biology at HMS, and the faculty will include Eric Jacobsen, Kirschner, Matthew Shair and Gregory Verdine, with Rebecca Ward as director of research affairs.

Traditional research approaches use gene mutation to discover how proteins function, but small cell-permeable molecules that bind to target proteins can also be useful, and may be as powerful as gene mutations. In

principle, ligands can cause any of the effects of genetic mutations but, at present, there are not enough ligands with known characteristics. "We believe the best way to find new protein ligands is to design libraries of synthetic molecules based on insights from a study of natural products known to bind to proteins. It is this equivalency of ligands and mutations that suggested the name 'chemical genetics'," states the institute's literature.

Research at ICCB will aim to discover large numbers of proteins that act as inhibitors, activators and gain-of-function ligands. The founders hope that "chemical genetics will become a major method for identifying gene function, as well as an important tool for drug discovery".

The institute will be a training ground where chemists and biologists can educate each other. It is expected to employ about 50 researchers and staff, half of them students and postdocs. There will be four fellows, each with a team of two or three researchers from backgrounds such as cell and molecular biology and synthetic organic chemistry. **B.H.**

Table Sites of internet interest for cell biology

Organizations and societies	
Federation of American Societies for Experimental Biology	www.faseb.org/
American Society for Cell Biology (placement)	www.faseb.org/ascb/place/place.htm
FASEB Consensus Conference on Graduate Education report	www.faseb.org/opar/educrpt.html
Commission on Professionals in Science and Technology (CPST)	www.aaas.org/opst/
Academic laboratories	
Institute for Chemistry and Cell Biology	www.hms.harvard.edu/iccb/
UC Berkeley Department of Molecular Cell Biology	mcb.berkeley.edu/
Corporations employment	
Hoerchst Marion Roussel	www.hmri.com
Eli Lilly	www.lilly.com
Incyte Pharmaceuticals	www.incyte.com
Grants and funding (private)	
The Pew Charitable Trust	www.pewtrusts.com/
Packard	www.packfound.org/packhome.htm
Burroughs Wellcome	www.bwfund.org/index.html
Howard Hughes Medical Institute	www.hhmi.org
American Cancer Society	www.cancer.org/grants/index.html
Vanderbilt Medical Center	www.mc.vanderbilt.edu/vumc/biosci/fundag.htm
Tram Research Funding Opportunities	tram.rice.edu/TRAM/fund/index.html
The Foundation Center	www.fdncenter.org

end of his graduate work at the University of California, San Francisco when he started to investigate the various options for using his degree. His scouting produced a collaboration in neurobiology with Regeneron of Tarrytown, New York. After several months of work in California, Bowen volunteered to continue the work at Regeneron for three or four months. The success of the project resulted in him doing a two-year postdoc at the company with George Yancopoulos.

Bowen warns that a postdoc in industry can vary considerably from one company to the next, and even within a company. "Through good planning and good fortune," he ended up doing projects that gave him creative control as well as experience in research. He advises people looking at postdocs in industrial research to establish whether the position will allow them to make decisions or whether they will be executing someone else's plan.

The FASEB report says: "If faculty retire soon after they reach 65, a significant number of jobs in academia is predicted for the next 10 years." However, as retirement is no longer mandatory at 70, the University of California at Berkeley offered an early retirement incentive scheme more than five years ago. This resulted in 30% of the faculty in molecular and cell biology retiring early. "Fortunately, we've been able to recycle most of those positions," says Schekman. Some universities are implementing similar schemes, including Cornell, North Carolina, Pittsburgh and Southern California.

Reasons for maintaining, or even expanding, faculty numbers include the projected increase in college enrolment from 14.2 million in 1995 to 16 million in 2005, and increased interest in the life sciences. According to Schekman, there is a nationwide trend showing that more stu-

dents are interested in medicine and biotechnology. At Berkeley, molecular and cell biology is the most popular undergraduate course, accounting for nearly ten per cent of students. □

Brendan Horton is on the staff of Nature, based in Washington. e-mail: b.horton@naturedc.com.

Favouring the brave

Potter Wickware

With US university science departments saturated and postdocs vigorously competing for the scanty number of faculty openings, does the discouraging proverb "Many are called but few are chosen" describe the lot of job-seekers in cell biology? "Not at all!", asserts Mina Bissell, director of the life sciences division at Lawrence Berkeley Laboratory in California and past president of the American Society for Cell Biology.

But many cell biology jobs are now in new areas: information management, computer science and engineering are becoming integral components of the discipline. High-resolution microscopy, for example, relies heavily on computational biology, a dynamic new subdiscipline rich with opportunity, yet the traditionally trained biologist may not have these skills.

Research problems are becoming so complex that collaborative projects are inevitable, Bissell observes. She believes that agencies such as the National Science Foundation, the space agency NASA and the Department of Energy should significantly complement the National Institutes of Health (NIH) by supporting interdisciplinary research. And universities' criteria for granting tenure must also change to reflect the newly collaborative nature of cell biology.

Are we training too many biologists? "We need all the talent we can get," Bissell emphasizes. She does not believe the gloomy prediction originating in the physics community that the end is nigh for growth in science ventures. "We have only scratched the surface. The Human Genome Project will be completed in 2005 and that will be just the beginning. Looking ahead we see magnificent problems that will keep us busy for decades."

But it must be acknowledged that certain opportunities are limited. The traditional route of academic tenure track at a research institution is a diminishing option. The path chosen by Michael Goldman is an example of the changing career landscape in cell biology. He works on X-chromosome inactivation and trains cell and molecular biologists at San Francisco State University. "When I left the ivory tower in 1988, one of my mentors said 'You're leaving the priesthood'. Not many people would put it that baldly," Goldman says, but it's undeniable that this mindset lingers on.

Goldman observes that in northern California, at least, the job market is strong. Masters and bachelors students are able to land technical jobs within four weeks as research assistants and scientists in the San Francisco Bay area's three main research universities, two large government labs or more than 500 biotechnology companies. The problem is not in placing students, but in ensuring that they complete their university courses, says Goldman. "We're not near the point of saturation in cell biology as a whole, although the academic faculty paid with hard money is at its limit." There are plenty of 'soft' money jobs, a trend that is likely to continue, particularly if the NIH fulfils its promise of increased funding in future.

Gerard Manning did research at Stanford and now develops genome analysis software at Molecular Applications Group, a bioinformatics company in Palo Alto, California. He agrees that the west coast job market is healthy: "There's lots of technician mobility. I've heard of good people getting multiple offers from the first few resumés. Personal contact always helps. In some fields there is an acute shortage — bioinformatics, people with gene-array chip experience." But he notes that cell biology is increasingly being defined by the ability to access, manipulate and interpret huge amounts of data.

How should cell biologists train in information management? It's easier said than done, says Joel Bellenson, who used his Stanford BA in biology and programming skills to co-found Pangea Systems, a bioinformatics company in Oakland, California. "Formalized training is hard because things are changing so fast." Nevertheless, the problems are fairly straightforward. "The DNA and protein patterns and the signalling pathways will always be there, as will the strategy of reasoning from observations derived from



Bissell: 'magnificent problems' ahead.

model organisms."

How should the individual contemplating a career in cell biology proceed? Concentrate on the present, Bissell advises. "Be flexible. If something comes up that you need to learn, learn it. But be excited

about science, not about employment. If you're truly excited, the job will follow. Your degree can prepare you for all sorts of eventualities that it's impossible to foresee. Start by doing research as an undergraduate, to see if it's what you enjoy." Goldman concurs. "If all you want is a job, maybe a BA or MA is good enough. But don't get a PhD unless you are sure. Then carefully reflect on whether you like the principal investigator, whether you are inspired by the research."

Manning warns that, for up to 12 years, someone doing a PhD followed by postdocs will make up to \$20,000 a year less than someone who takes a technical job straight after an undergraduate degree. As Bissell says, "The moment of discovery when you develop a gel, see the excitement in a pupil's eye — these are the reasons we do science. It has never been easy to do science well and have security and recognition from the start." □

Potter Wickware is a science writer in Oakland, California, USA.

e-mail: wick@netcom.com

Not enough places to go in Europe

Owen Goldring

How should an undergraduate starting a degree in biological sciences find out about a research career in cell biology? Imagine a seminar, "Starting to put the cell biology timepiece together", at which the speakers explain their concept of cell biology, describe what training is best, and what salaries can be expected.

The main difference between cell biology and other areas of biology is that the cell biologist is not confined to particular cells, says Colin Hopkins, head of the Medical Research Council Unit of Molecular Cell Biology at University College London. Hopkins defines molecular cell biology as the study of how molecules combine to create macromolecular assemblies within cells, and how those assemblies — ribosome, nuclear pore, and so on — are kept working in concert.

Hopkins believes that the molecular mechanisms in all cells will turn out to be similar, and therefore would not be surprised to find a cell biologist working on T cells one day and on nerve cells the next. Or they might use their skills in research on gene-

knockout functional analysis (reverse genetics) in yeast, nematode, *Drosophila* or transgenic mouse models, working out the fate map of a protein.

Apoptosis, a process at the other end of the spectrum in which the cell's machinery unravels in an ordered, programmed death, is discussed by Roberto Solari, head of cell biology at Glaxo Wellcome in Stevenage, southern England. Solari describes how adenoviral E1B protein inhibits apoptosis. Researchers in Solari's group, after doing yeast two-hybrid experiments, found that E1B bound to the human protein Bak. In this instance, Bak — an 'executor' of apoptosis —

has been inhibited by this viral E1B product (see *Nature* 374, 731–733; 1995).

Hans Geuze, of the department of cell biology in the medical faculty of Utrecht University in the Netherlands, has been localizing MHC class II molecules to various intracellular organelles during the cellular processes of antigen presentation. This research, soon to be reviewed in *Immunology Today*, is a classic example of how immunoelectron microscopy, biochemistry and molecular biology have all come together at the level of the cell. "Cell biology is an integrating discipline," says Geuze.

Staffan Normark, vice-dean of research at

Salaries and qualifications across Europe

To see what general advice on cell biology is available in Europe, access <http://www.ukplus.co.uk/dynamic/index.html>, which leads to the search engine UK Plus. Type in "cell biology", and select "in all of the Web". On its next page, UK Plus offers various countries. Type in "cell biology" again, with a country.

The Netherlands

Four-year degree, followed by four- to six-year PhD (salary 27,000 to 48,000Gld (US\$13,170 to \$23,420)). Grants are for four years. Postdoc salary is 49,000 to 59,000Gld over four years. Most Dutch PhDs do their postdocs in Holland. About 5% of postdocs get permanent academic positions. Geuze estimates that over the past 30 years more than 70% of the postdocs from his lab have gone into the pharmaceutical industry and the rest into teaching or scientific publishing.

France

Four-year degree, followed by the 'troisième cycle' (PhD programme). The first year is a seminar- and project-based introductory programme with an examination, followed by a 'state fellowship' for the next three years (salary FF72,000 (US\$11,810) net of tax). Competition is intense. After this, most French postdocs go abroad because the government does not fund PhD graduates to do postdocs in France. Initially, fewer than 10% of returning postdocs will find permanent jobs in academic research (salaries start at about FF120,000 net of tax). The government is talking about creating state-funded postdoc positions for returning postdocs. Louvard says that, without drastic action, many of the best students will opt for other professions.

Sweden

Three-year degree, followed by a master's programme (non-taxable stipend of Kr8,000 (US\$985) a month) and a PhD, possibly funded via the supervisor's grant. The Karolinska Institute in Stockholm has 1,600 registered graduate students, of whom 500 are engaged

in biomedical research. Sweden is creating 'graduate schools in biomedical research' for 450 students at various universities. Many Swedes do postdocs elsewhere in Europe on European Molecular Biology Laboratory (EMBO) fellowships or in the United States. Some private Swedish grant foundations give funds for two years abroad and then three at home. Swedish postdoc salaries are around Kr20,000 a month (taxable). There are few permanent jobs. Staffan Normark says: "The Karolinska plans to start its own postdoctoral programme."

Italy

Five-year degree; four-year PhD. Annual salary 18 million to 24 million Lira (US\$10,020 to \$13,370). Meldolesi, of DIBIT in Milan, has taken the unusual step of registering many of the institution's PhD students with the UK Open University. "Unfortunately, in Italy, there are PhD courses given by people who are not qualified to do so, and there's sometimes little quality control," he says. Italian postdocs are encouraged to go abroad via the EC or EMBO fellowships. In Italy, postdocs get about 30 million Lira a year. Returning postdocs face the familiar problem of few permanent jobs.

United Kingdom

Three- or four-year degree and three-year PhD (salary about £9,000 (US\$14,690)). A few PhDs are funded for four years (salary £10,000–12,000). Such courses are competitive. The 'lead year' is spent rotating through various labs, or with a short spell in industry, followed by a three-year programme of research. Hopkins would like this four-year programme to become the norm. Postdocs are well-placed for short-term positions (salary about £18,000 for a new postdoc) because of charity funding, but not for permanent jobs in academic or government research institutions, which eventually pay £30,000–£40,000. Hopes that industry will help may not be realized because the major pharmaceutical companies are reducing their workforces following mergers.

O.G.



King: favours early introduction.

the Karolinska Institute in Stockholm, is working on the use of microbes or microbial products to study cellular processes (see *Science* 271, 315–316; 1996). An example is how *Listeria* can move inside cells and polymerize actin. “The opportunities for cellular microbiology are enormous — it brings you to the heart of molecular pathogenesis,” says Normark.

Everyone concurs that the integration of molecular biology techniques into cognitive neurobiology is one exciting area, and that the integration of cell biology into a study of pathogenesis at the tissue level is another.

Daniel Louvard, director of the research division of the Institute Curie in Paris, produces an analogy that defines cell biology and captures the flavour of the excitement and opportunity: “Imagine you are a visitor from Mars and you come across a sixteenth-century timepiece, in bits. All those wheels and springs. You know what the concept of ‘time’ is, but you have all these wheels and things in front of you — it would take you a long time to put it together so it works.”

Jacopo Meldolesi is scientific director of DIBIT, the internationally acclaimed basic research branch of the San Raffaele Institute, which hosts the labs of various Milan University professors. He would not rule out a medical degree followed by specialization as a way in to cell biology. Solari, Geuze and Normark suggest starting with a good biological degree (covering molecular biology and genetics), and seek to encourage curiosity about the “culture of biology”.

Rod King, director of studies at the National Institute for Medical Research in London, agrees: “I think it’s important to introduce students to biology early on, and then they don’t get afraid of it, because I don’t think there’s any doubt that some chemists and physicists never cotton on to biology. They’re not attracted, or it remains a pile of nomenclature which is foreign. We have programmes here, for example, which are involved in using quite sophisticated chemistry to construct ‘caged’ molecules.”

Louvard goes further: “On top of molecular genetics, we need more graduates who understand quantitative biochemistry — we also need chemists and physicists to go into cell biology.” And Hopkins says: “If I were setting out on a career now, I’d want to have an understanding and a technology that was flexible enough to take me into nerve cells, or the immune system, or whatever. You need to be a card-carrying molecular biologist. You need to know how to manipulate DNA when you are an undergraduate.

“So, if you’re coming to university and you want to be a cell biologist, you should

make sure you have a sound education in biochemistry and a good education in molecular biology.” You will then need a PhD (see box) and, as King advises, “remember, you’re very much part of a team”.

As with other disciplines, the ‘postdoc run’ follows the PhD. Short-term postdoc contracts are reasonably easy to find (see box), but permanent jobs are another matter. European governments must give urgent thought to the creation of more permanent jobs for experienced postdocs. Molecular cell biology is the key to biomedical research



Louvard: captures the excitement.

in all the main health-care problems of the next millennium — including neurodegenerative disease, atherosclerosis and cancer. If the most talented of Europe’s young researchers are going to be attracted into, and kept in, academic science, governments need to plan for the future.

How much money will these governments have wasted in training people to the end of a second postdoc, only to see them dropping out of mainstream science because there is no career structure? □

Owen Goldring is a UK-based freelance science/medical writer and industrial consultant. e-mail: ogoldring@edit1.demon.co.uk.

Opportunities open up in Dresden

Barbara Miller

Long after political reunification in 1990, Germany is still struggling to achieve economic and social uniformity. Scientific research in east Germany is developing frustratingly slowly, but will receive a welcome boost with the foundation in Dresden of a Max Planck institute for cell biology and genetics, due to open in mid-2000. The directors are Kai Simons, Anthony Hyman and Marino Zerial from the European Molecular Biology Laboratory (EMBL) in Heidelberg and Wieland Huttner of the University of Heidelberg.

One of the fundamental principles of the Max Planck Society, Germany’s main scientific research organization, is that its institutes are evenly distributed throughout the country. After reunification, institutes and departments were closed in the west to pay for 20 planned institutes in the east. But progress has not been straightforward. An institute needs a strong local scientific environment. Sadly, standards of research in east German universities are in general lower than in the west.

The fact that the new institute is in historic Dresden rather than in its rundown

industrial neighbour Halle will help recruitment, according to Huttner. In Dresden there are relatively few research institutes, mainly concerned with physical sciences. But the small biology faculty at the local Technical University is expanding and will collaborate with the new institute.

East Germany does not have the western tradition of collaborative scientific research. “Combining molecular disciplines like cell biology and genetics in the east did not take place,” says Simons. “The most difficult problem will be to attract the best scientists.” West German scientists avoid the east because of this historical image. Jürgen Kirschner, director of the Max Planck institute for microstructure physics in Halle, says he has mainly recruited from other countries, particularly Asia. “The image problem will decrease with time,” says Kirschner, “but will last at least the next ten years.”

The Dresden institute will have a collaborative research programme aimed at elucidating the molecular mechanisms of tissue formation. The main individual projects will be on cell division, structure of cell organelles, membrane transport and cell polarity. The institute will also develop biochemical and microscope techniques in collaboration with the Technical University. Herwig Gutzeit, vice-dean of biology, says: “The university expects to get a large impetus to its own research from the collaboration.”

Dresden has always been an important bridge between east and west. “Unified Europe still needs this gateway,” says Simons, so the institute will train young scientists from eastern Europe using money from the European Union.

Simons plans to give young scientists a central role in the work of the institute: the relatively small directors’ research groups will interact with 19 scientifically independent junior groups. The concept of young scientists as group leaders is revolutionary in Germany.



Simons: not curbed by tradition.

Simons has experienced the advantages of his proposed system while at EMBL, which, as a European rather than a German institution, is not constrained by national traditions.

“The young group leaders change because they are hired on fixed-term contracts [for a maximum of five years] and therefore the groups are always filled with innovative ideas and enthusiasm.” Recruitment of group leaders is due to start soon, and the institute will soon hold the first in a series of annual symposia. Further information can be obtained from: dresden@embl-heidelberg.de □

Barbara Miller can be contacted via Alison Abbott in Nature’s Munich office. e-mail: a.abbott@nature.com.